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Stimulation of Ciliary Activity by Serotonin in Isolated Ciliated Cells from *Helisoma trivolvis* Embryos: Involvement of Cyclic AMP, Ca^{2+}, and Protein Kinase C

by

Kimberley Jean Christopher

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Physiology and Cell Biology
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K. Christopher

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10119 - 84 Avenue
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University of Alberta

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Dr. J. I. Goldberg (Supervisor)

Dr. J. P. Chang (Committee Member)

Dr. B. K. Mitchell (Committee Member)

Dr. P. A. Smith (External Examiner)

Aug. 21, 1997

Dept. of Biological Sciences
University of Alberta

CW-405 Biological Sciences Centre
Edmonton Alberta T6G 2E9 Canada

Jeffrey I. Goldberg:

Phone: (403) 492-1272 Fax: (403) 492-9234
E-mail: jeff.goldberg@ualberta.ca

Faculty of Graduate Studies and Research
University Hall
University of Alberta
Edmonton, Alberta
T6G 2E9

August 28, 1997.

Re: Ms. K.J. Christopher M.Sc. thesis

To whom it may concern:

As supervisor of Kim's graduate program, we co-authored 2 papers that were published in international journals and 3 abstracts of the annual meeting of the Society for Neuroscience. All the data in these publications result from Kim's original thesis research, and I give my full permission that these data be included in her M.Sc. thesis.

Sincerely,

Jeffrey I. Goldberg, Ph.D.,
Professor

Dept. of Biological Sciences
University of Alberta

CW-405 Biological Sciences Centre
Edmonton Alberta T6G 2E9 Canada

Kevin Young

Phone: (403) 492-5494 Fax: (403) 492-9234
E-mail: kyoung@gpu.srv.ualberta.ca

August 29, 1997

Faculty of Graduate Studies and Research
University Hall
University of Alberta
Edmonton, Alberta
CANADA

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Dear Sir/Madame,

This is to inform you that Kim Christopher has my permission to use data from any publications which I have contributed to for use in her MSc. thesis. Kim has helped to supervise me, as an undergraduate student, on a project which contributed some information to her thesis and to one submitted paper (Stimulation of ciliary activity by 5-HT is mediated by a unique isoform of protein kinase C in *Helisoma trivolvis* embryos) and to one Society for Neuroscience abstract (Serotonin-stimulated cilio-excitation is mediated by a phorbol ester-insensitive isoform of PKC in embryonic ciliated cells from *Helisoma trivolvis*).

Kevin Young

Department of Biological Sciences **University of Alberta**
Biological Sciences Centre CW405 **Edmonton Alberta T6G 2E9 Canada**

Telephone: Departmental Office: (403) 492-3308; John P.Chang: (403) 492-1278 / 492-1275
FAX: Departmental Office: (403) 492-9234; E-mail: John.Chang@ualberta.ca

28 August, 1997

Faculty of Graduate Studies and Research
University Hall
University of Alberta
Edmonton, Alberta
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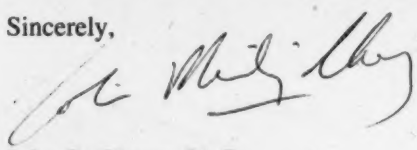
Dear Sir/Madame,

Ms. Kimberly J. Christopher has the right (and my permission) to include data published in Christopher, Chang and Goldberg (1996, J. Experimental Biology, Vol. 199: 1105-1113) and Christopher et al. (1997; Society of Neuroscience Abstracts, in press) as part of her thesis work. All the data result from her original research work. I am a member of her supervisory committee and I have worked closely with her supervisor, Dr. Jeffrey I. Goldberg on several projects. My contribution to Ms. Kim Christopher's publications is mainly as an advisory role on the experimental design and the use of drug/vehicle controls. I have also acted as interim supervisor to Ms. Christopher for part of her research project work. If more information is required, please do not hesitate to contact me.

Sincerely,



John P. Chang, Ph.D.
Professor.

One can't be satisfied with just
looking at so wonderful a structure:
chiefly because one can't get clear
how such an unbelievable
motion is brought about...

- one of the earliest descriptions of ciliary motility
Antony Van Leewenhoek, June 28, 1713

ABSTRACT

During development, embryos of the pulmonate gastropod, *Helisoma trivolvis*, display a cilia-driven rotation behavior that is regulated by the neurotransmitter serotonin. The present study examined the signal transduction mechanisms underlying serotonin-mediated cilio-excitation in cultured ciliated cells from *H. trivolvis* embryos. Using time-lapse videomicroscopy, ciliary beat frequency (CBF) was measured in response to various pharmacological manipulations. Serotonin increased CBF in a concentration-dependent, reversible manner. Treatments known to elevate intracellular cyclic AMP concentrations did not affect basal or serotonin-stimulated CBF. In contrast, the cilio-excitatory effect of serotonin was mimicked by treatments that increase intracellular Ca^{2+} concentrations, and blocked by treatments that prevent extracellular Ca^{2+} influx. Furthermore, diacylglycerol analogs transiently increased CBF, whereas protein kinase C (PKC) inhibitors blocked serotonin-induced cilio-excitation. Application of phorbol esters did not affect CBF. These results suggest that an influx of extracellular Ca^{2+} and activation of a phorbol ester-insensitive, diacylglycerol-sensitive PKC isoform are signal transduction components in serotonin-stimulated ciliary beating.

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List of Abbreviations

5-HT.....Serotonin
AA.....Arachidonic Acid
ACh.....Acetylcholine
AHS.....Antibiotic-containing *Helisoma* saline
ATP.....Adenosine 5'-triphosphate
CaM.....Calmodulin
cAMP.....3', 5'-cyclic adenosine monophosphate
CBF.....Ciliary Beat Frequency
cGMP.....3', 5'-cyclic guanosine monophosphate
DA.....Dopamine
DAG.....Diacylglycerol
DIC.....Nomarski Differential-Interference-Contrast
DiC_8.....*sn*-1,2-dioctanoylglycerol
ENC1.....Embryonic Neuron C1
Fura-2-AM.....Fura-2 acetoxy-methyl ester
HDM.....*Helisoma* Defined Medium
HS.....*Helisoma* Saline
HSD.....*Helisoma* Saline with Dextrose
IBMX.....3-isobutyl-1-methylxanthine
IP_3.....Inositol 1,4,5-*tris*phosphate
OAG.....1-oleoyl-2-acetyl-*sn*-glycerol
PDBn.....Phorbol 12β, 13α-dibenzoate
PKA.....Protein Kinase A
PKC.....Protein Kinase C
PLA_2.....Phospholipase A_2
PLC.....Phospholipase C
PLD.....Phospholipase D
SC-9.....*N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide
TFP.....Trifluoperazine
TPA.....Phorbol 12-myristate 13-acetate

INTRODUCTION

Ciliary activity is one of the most common, widespread cellular phenomena known. Cilia are membrane-bound hair-like projections found in unicellular organisms, several plant species, and almost every animal species. Locomotion, respiration, feeding, and reproduction are examples of the physiological functions that depend on ciliated epithelial cells (Witman, 1990). Control of these physiological actions of cilia can occur through a variety of stimuli, including chemical (*i.e.* neurotransmitters), mechanical, hormonal, and electrical. Although several signal transduction pathways have been implicated in the intracellular regulation of ciliary activity, the function of any signal transduction pathway in mediating a neurotransmitter response has not been well established.

During development, early embryos of the pulmonate gastropod, *Helisoma trivolvis*, display a cilia-driven rotation behavior that is in part modulated by the neurotransmitter 5-HT (Diefenbach *et al.*, 1991). Whereas 5-HT has been established as a regulator of ciliary beating in this system, as well as others, the signal transduction pathways used are not well understood. The isolation and identification of single cultured ciliated cells from *Helisoma* embryos permit a direct examination of ciliary activity in response to a variety of pharmacological manipulations. The main objective of this thesis is to examine the role of the cyclic AMP, Ca^{2+}, and protein kinase C (PKC) intracellular signaling pathways in mediating 5-HT-stimulated ciliary beating in primary cultures of *Helisoma* embryonic ciliated cells.

I. Cilium Structure

Cilia are hair-like projections of cells surrounded by a contiguous extension of the cell's plasma membrane. Motile cilia move with an asymmetric beat, consisting of an effective stroke (also known as power stroke) and a recovery stroke (Witman, 1990). The

planar effective stroke is initiated by a bend at the base of the cilium, causing the upright cilium to whip parallel to the cell surface. The effective stroke is a relatively quick movement, taking only one-third of the total duration of the ciliary beat (Sleigh and Barlow, 1982). Due to this quick motion, in addition to the upright position of the cilium, the resultant effect of the effective stroke is to move the surrounding medium in the same direction as the movement of the cilium. The recovery stroke returns the cilium to its original position through a complex 3-dimensional motion that is propagated as a base-to-tip bend in the ciliary shaft (Witman, 1990; Sleigh and Barlow, 1982). Making up the cilium is an abundance of tubulin and non-tubulin polypeptide structures, collectively known as the axoneme. These structures include the characteristic "9+2" arrangement of doublet microtubules, inner and outer dynein arms, interdoublet (nexin) links, and radial spokes and spoke heads (Figure 1).

The axoneme is an evolutionarily conserved structure with a characteristic "9+2" arrangement: a central pair of singlet microtubules is surrounded by 9 outer doublet microtubules (Witman, 1990). The central microtubules are connected to one another by a central bridge. Two pairs of projections extend from the singlets, also known as the central sheath, as they appear in micrographs to surround the central pair (Figure 1); the projections repeat every 16 nm along the length of the central pair. The 9 outer doublets are each made up of an A tubule and a B tubule (Figure 1). The singlet tubules of the central pair and the A tubules are composed of 13 protofilaments, whereas the B tubule consists of 10 or 11 protofilaments, depending on the species examined. Each protofilament is 4 nm in length and is made up of α- and β-tubulin heterodimers that repeat every 8 nm along the length of the protofilament (Witman, 1990). In some cases, there exists a connection between the 2 outer doublets that lies within the plane of the ciliary beat. However, this connection appears to be species-specific and is not always observed (Sleigh and Barlow, 1982).

The inner and outer dynein arms extend from the A tubule of one doublet towards the B tubule on the adjacent doublet (Figure 1). The dynein arms are the "powerhouse" of the ciliary beat due to the inherent ATPase activity within the heavy chains. The outer dynein arms occur every 24 nm along the length of the A tubule. They consist of 3 heavy chains, accompanied by several intermediate and light chains (terminology based on molecular weight). In contrast, the inner dynein arms are found in 2 configurations: a dyad or a triad, consisting of 2 or 3 large globular domains, respectively. Each globular domain is made up of 4 or 5 heavy chains, in addition to several intermediate and light chains. A triad/dyad/dyad sequence of inner dynein arms repeat every 96 nm along the length of the A tubule (Witman, 1990).

Nexin, or intermediate filaments, occur every 96 nm along the length of the outer doublets connecting the A tubule of one doublet to the B tubule of the adjacent doublet (Figure 1). Although the definitive function of nexin is unknown, it has been suggested that it could play a role in the sliding mechanism involving the dynein cross-bridge system (Satir, 1992). Furthermore, nexin may function to support the axoneme or could act as an internal source of resistance, required for the characteristic bending wave of the cilium (Walczak and Nelson, 1994). In *Tetrahymena* cilia, the Ca^{2+}-binding protein calmodulin is present in the longitudinal axis of the outer doublets and possibly interacts with nexin, suggesting that nexin may be involved in Ca^{2+}-dependent processes modulating activity of the ciliary beat (Ohnishi *et al.*, 1982).

The radial spokes, terminating in spoke heads, extend from the A tubules towards the central pair and repeat every 96 nm along the length of the cilium. Similar to the interdoublet links, the actual function of the radial spokes and spoke heads is unknown. However, it has been suggested that they play a role in the initiation and maintenance of the ciliary beat (Witman, 1990).

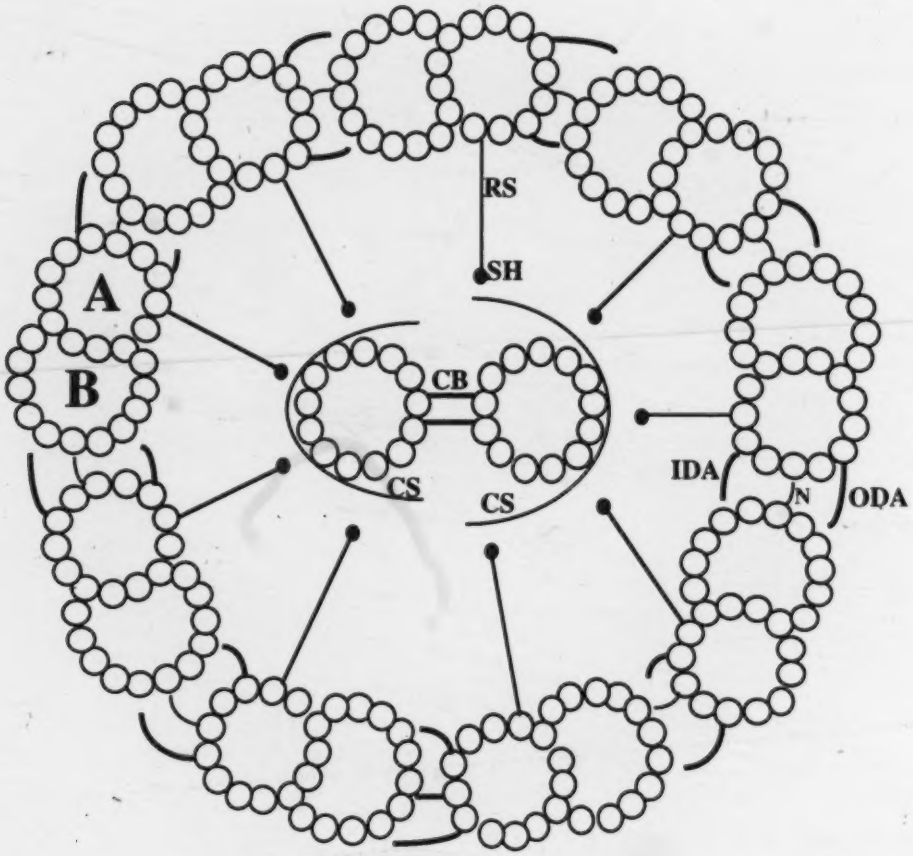


Figure 1: Schematic representation of a cross-section of the axoneme. The characteristic "9+2" microtubule arrangement, consisting of 9 outer doublets surrounding a pair of singlet microtubules, and the other microtubule-associated structures are shown. Abbreviations: A and B, A- and B-tubules of the doublet microtubule, respectively; CB; central bridge; CS, central sheath; ODA, outer dynein arm; IDA, inner dynein arm; N, nexin; RS, radial spoke; SH, spoke head. Adapted from Witman, 1990.

II. Sliding Microtubule Model

Experiments utilizing isolated axonemes have laid the foundation for the sliding microtubule model of ciliary beating. Isolated axonemes, when stimulated to move by addition of ATP, will elongate, due to the "walking" or sliding of one doublet (N) along adjacent doublets (N+1; Sale and Satir, 1977). The sliding doublet always moves towards the distal end of the cilium (Sale and Satir, 1977). Based on these observations, the mechanochemical cycle of the ciliary beat was proposed by Satir *et al.* in 1981 (see Figure 2). When inactive, the dynein arm, which is permanently attached to the A tubule of doublet N, stretches across the interdoublet gap (21 nm) and attaches to a specific binding site on the B tubule of the N+1 doublet (Figure 2-I). This position of the dynein arm is referred to as "rigor". Upon addition of ATP, the dynein arm shortens and releases from the B tubule of the N+1 doublet, leaving a 10 nm interdoublet gap (Figure 2-II). This step appears to be independent of ATP hydrolysis, but is dependent on the presence of ATP. Following release, the dynein arm re-extends 40° basally towards the B tubule of N+1 (Figure 2-III) and re-attaches (Figure 2-IV). Re-extension and re-attachment are accompanied by ATP hydrolysis and release of the products of hydrolysis, ADP+Pi, respectively. Following re-extension and reattachment, the dynein arm returns to the "rigor" position to resume equilibrium, causing the N+1 doublet to slide distally relative to the N doublet (Figure 2-V). It is in this active manner that sliding of the doublets along one another occurs. The return of doublet N+1 to its original, resting position occurs through passive interactions between the 2 doublets when the dynein arms are not active (Satir, 1992). Coordinated beating is believed to be due to sliding occurring on one half of the axoneme (effective stroke), followed by differential sliding on the other side of the axoneme to return the cilium to its original position (recovery stroke). In order for beating to occur in such a manner, one half of the axoneme must be in an active, cycling state, while the opposite half is in an inactive, non-cycling state; this is known as the Switch

Point Hypothesis (Satir and Matsuoka, 1989). Evidence for this hypothesis has been demonstrated using arrested permeabilized cell models. Trypsin-treated, isolated, permeabilized axonemes split in half upon application of ATP, as the active (*i.e.* attached) doublets split apart from the inactive (*i.e.* unattached) doublets that are free to slide along one another (Satir and Matsuoka, 1989).

III. Microtubule Sliding Produces Bending

The sliding microtubule model proposes a way of movement between the doublets, but the question remains as to how the sliding motion is transformed into the characteristic bend of cilia. The answer lies in the internal resistances generated by interactions between various axonemal components. Radial spoke - central sheath interactions are involved in cilia bend formation (Satir, 1992), as cilia mutants without central microtubules or radial spokes are unable to produce characteristic bends. Additionally, circumferential nexin links between adjacent microtubule doublets (Satir, 1992; Walczak and Nelson, 1994), the connection between doublets through dynein arms (Witman, 1990), and the interactions between dynein and the overlying plasma membrane through an amorphous link (Witman, 1990), have all been proposed to produce resistive forces capable of transforming sliding into bending. As these resistance factors constrain the structural integrity of the axoneme, the act of sliding is transformed from a linear vector into a non-linear movement, which is the characteristic bend observed (see Figure 2-VI; Cosson, 1992; Walczak and Nelson, 1994).

Although the precise mechanisms underlying the regulation of the movement of the cilia are unknown, several lines of evidence suggest possible interactions between individual axonemal components. The central pair is in close association with the radial spokes and spoke heads; the timing and location of this interaction is directly correlated with dynein arm movement, suggesting a role for these axonemal components in

regulation of the ciliary beat (Witman, 1990). Further, the central pair rotates 360° for every beat cycle; it has been proposed that this rotation may coordinate sliding between doublet microtubules upon dynein activation (Witman, 1990). This coordination may also involve the interdoublet (nexin) links (Walczak and Nelson, 1994). However, much more work is required to fully understand the mechanisms involved in converting the sliding microtubules into a characteristic ciliary beat cycle.

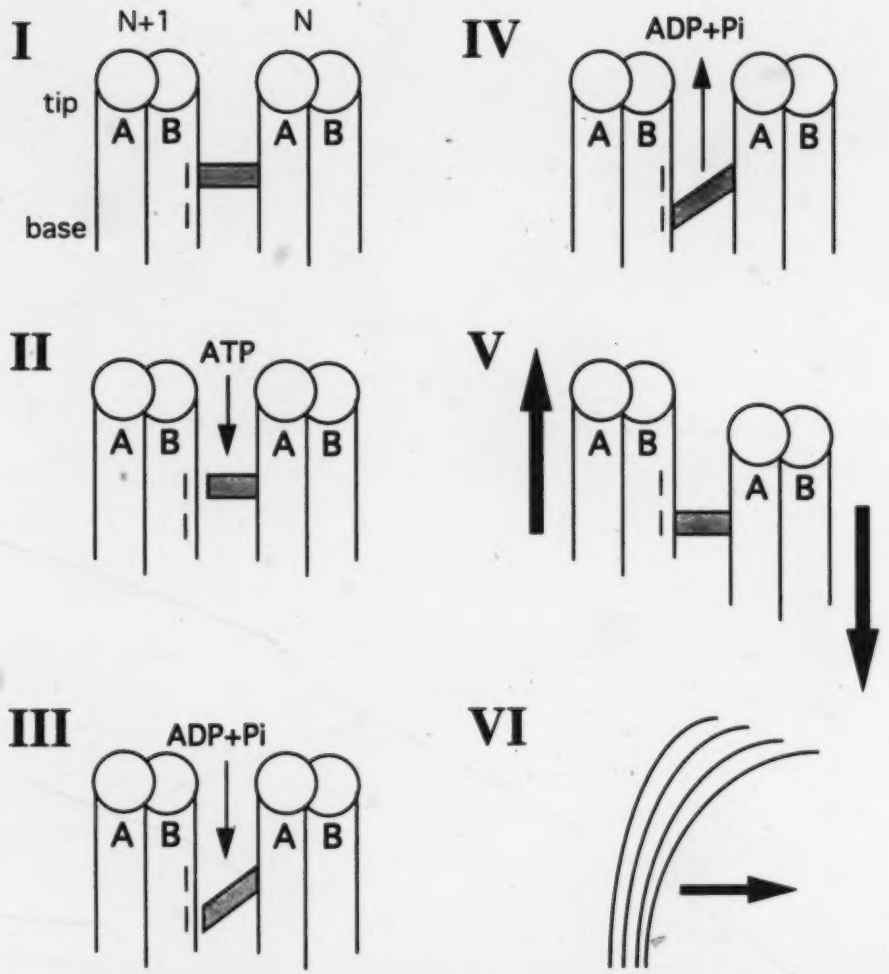


Figure 2: Schematic representation of the mechanochemical interactions between dynein arms and microtubule doublets. (I) Rigor state of dynein arms (shaded). (II) Dynein arm releases from the B tubule of the N+1 doublet in the presence of ATP. (III) Re-extension of dynein arm basally by 40° towards N+1 doublet; ATP hydrolysis required. (IV) Re-attachment of dynein arm to N+1 doublet, concurrent with release of the ATP hydrolysis products, ADP+Pi. (V) Doublet N+1 slides tipwards and doublet N slides basally. (VI) Resistances inherent in the cilium cause a resultant bend towards the right. Adapted in part from Satir *et al.*, 1981; Satir, 1982.

IV. Comparative Analysis of Ciliary Activity

In general, 3 forms of ciliary regulation have been identified: stimulation, arrest, and reversal. Stimulation, an increase in ciliary activity with no change in the direction of the effective stroke, occurs in actively beating cilia or quiescent cilia. Arrest is defined as the cessation of ciliary movement, usually with the cilium being halted at the end of the recovery stroke (Murakami, 1989). Ciliary reversal involves a 180° shift in the direction of the effective stroke (Murakami, 1989). Reversal may or may not involve an increase in the beat frequency.

The gill of the marine bivalve *Mytilus edulis*, which contains several groups of cilia, has been one of the most important model systems for studying ciliary regulation. The lateral and abfrontal gill cilia are commonly studied due to their differential regulation of ciliary beating. The neuronally controlled, continuously beating lateral cilia produce the water current necessary for feeding in this animal. However, when aberrant food particles come in contact with the lateral cilia, the cilia momentarily arrest, thereby removing the particles from the gill (Murakami and Machemer, 1982). In contrast, as a second line of defense, mechanical stimulation of the abfrontal gill cilia, which are not neuronally controlled, transiently increases the beat frequency, again acting to remove food particles out of the gill (Good *et al.*, 1990). Thus, mechanical stimulation causes both ciliary arrest and ciliary stimulation within a single organ to modulate feeding behavior in *Mytilus*.

Similar to abfrontal gill cilia, ciliary stimulation has been demonstrated in numerous systems studied to date. In mammals, increased ciliary activity is observed in response to a variety of stimuli. However, ciliary reversal and ciliary arrest have not been observed in mammalian systems, but, as these studies concentrate on respiratory cilia, arrest or reversal would not be advantageous (Lansley *et al.*, 1992). In invertebrates, stimulation of cilia has been observed in numerous systems, such as the foot epithelium

of the gastropod molluscs *Tritonia diomedea* and *Lymnaea stagnalis* (Audesirk *et al.*, 1979; Syed and Winlow, 1989). Increased forward swimming in posteriorly stimulated protozoans is yet another example of ciliary stimulation (Bonini *et al.*, 1991).

Ciliary reversal normally elicits backwards swimming or modifies feeding currents (Murakami, 1989). For example, in *Paramecium*, as an avoidance response, ciliary reversal occurs upon anterior mechanical stimulation of forward swimming organisms, resulting in backward swimming (Bonini *et al.*, 1991). A similar behavior is observed in sea urchin pluteus larvae (Mogami *et al.*, 1992).

Ciliary arrest underlies a variety of behavioral modifications, such as migration, protection, feeding, and larval settlement. Arrest responses are observed in the gastropod veliger *Calliostoma ligatum* (Arkett *et al.*, 1987). This response may be involved in regulating diurnal migration or may act as a defense mechanism. In addition, cilio-inhibition, defined as slowing of the beat frequency, may be a modification of feeding behavior allowing the organism to remain in a zone of abundant food (Arkett *et al.*, 1987). Filter-feeding animals, such as bivalves, use ciliary arrest when the ciliated gill epithelium comes in contact with particulate matter (Murakami and Machemer, 1982). Non-optimal sized food particles are thus restricted from entering the oral cavity. In molluscan veligers, ciliary arrest is a main component of settlement behavior; when an appropriate developmental substrate is located, the ciliated velum stops beating, allowing the organism to sink within the water column to the preferred substrate (reviewed in Chia *et al.*, 1984).

V. Neurotransmitters Involved in Ciliary Regulation

Neuronal control of ciliary activity was demonstrated over 2 decades ago, through anatomical and physiological analyses (Mackie *et al.*, 1976). Since this discovery, it has been determined that the main neurotransmitter involved in ciliary control is serotonin (5-

HT), although dopamine, noradrenaline, and acetylcholine have also been shown to regulate ciliary activity.

Cilio-excitatory effects of 5-HT through innervation has been established in a variety of species. For example, activity in the lateral gill cilia of the marine bivalves, *M. edulis* and *Crassostrea virginica*, is modulated by release of 5-HT from the branchial nerve (Paparo and Murphy, 1975a; 1975b; Paparo, 1986). Likewise, activity in the ciliated foot epithelium of *Lymnaea stagnalis* (Syed and Winlow, 1989) and *Tritonia diomedea* (Audesirk *et al.*, 1979) are under the control of serotonergic pedal ganglion neurons, the pedal A-cluster neurons and Pd 21 neurons, respectively. In addition to neuronal innervation, synthesis of 5-HT by the tissue itself has been demonstrated. In frogs, 5-HT-stimulated mucociliary transport may be in part mediated by the synthesis of 5-HT within the palatine mucosa (Maruyama *et al.*, 1984). In several of these examples, 5-HT has been shown to have excitatory effects when applied exogenously. However, definitive evidence demonstrating that 5-HT is an endogenous factor present in the organism has not always been provided. For instance, addition of 5-HT modulates forward swimming in pluteus larvae of the sea urchins, *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus* (Mogami *et al.*, 1992), and ciliary activity in the larval form of another species of sea urchin, *Psammechinus miliaris* (Soliman, 1983), but evidence that 5-HT is found endogenously has not been presented.

Dopamine (DA) is more commonly a cilio-inhibitory neurotransmitter, as compared to the excitatory effects of 5-HT. In many cases where innervation has been firmly established, 5-HT and DA act as antagonistic neurotransmitters, exciting and inhibiting ciliary activity, respectively. For instance, branchial nerve innervation of the lateral gill cilia of *M. edulis* (Catapane, 1983; Paparo and Murphy, 1975a) and *Crassostrea virginica* (Paparo, 1986) inhibits ciliary activity though the release of DA. Additionally, DA-containing cells have been identified in the frog palatine mucosa,

where DA causes cilio-inhibition (Maruyama *et al.*, 1983). DA has also been shown to have cilio-inhibitory effects when applied exogenously in other systems, but the physiological relevance of these responses are unknown. For instance, ciliary reversal by DA, as indicated by increased backwards swimming, is seen in pluteus larvae of the sea urchin *P. depressus* (Mogami *et al.*, 1992). In contrast to these inhibitory and reversal effects, stimulation of ciliary activity occurs in early gastrula of the sea urchin, *P. miliaris* upon addition of DA (Soliman, 1983). Again, the physiological relevance of such an effect is unknown.

Noradrenaline is another neurotransmitter implicated as both an inhibitor and stimulator of ciliary activity. Release of noradrenaline from sympathetic nerve endings inhibits and stimulates ciliary activity in frog palatine mucosa in a concentration-dependent manner (Maruyama, 1984). However, these opposing actions are most likely due to inherent negative feedback interactions involving second messenger pathways, such as cyclic AMP and arachidonic acid metabolites (Maruyama, 1984). β-adrenergic agonists stimulate ciliary activity in rabbit tracheal epithelial cells, whereas a β-adrenergic antagonist blocks this cilio-stimulatory response. These data strongly implicate an adrenergic pathway controlling ciliary activity in rabbits (Verdugo *et al.*, 1980; Lansley *et al.*, 1992). Adrenergic receptor agonists excite the trochal cilia, and thus swimming behavior, in the trochophore larvae of the polychaete *Spirobranchus giganteus* (Marsden and Hassessian, 1986). The swimming behavior is inhibited in the presence of adrenergic receptor antagonists, suggesting the presence of an endogenous adrenergic pathway in this organism (Marsden and Hassessian, 1986). Similar cilio-excitatory effects of noradrenaline are apparent in the sea urchin, *P. miliaris* (Soliman, 1983); however, as before, the physiological relevance of this effect is unknown.

Acetylcholine also displays cilio-regulatory effects in numerous systems. For instance, ciliary arrest of the stigmatal cilia in the branchial basket of *Chelyosoma*

productum (Ascidiacea) is controlled by "ciliary arrest" neurons. These neurons have been postulated to form cholinergic neurociliary synapses (Arkett, 1987). Additionally, the ciliary arrest response in the ascidian *Ciona intestinalis* may be mediated by cholinergic synapses from an identified neuron in the central ganglion (Arkett *et al.*, 1989). Excitation of the parasympathetic nerve innervating the frog palate increased ciliary activity through the release of acetylcholine (Aiello *et al.*, 1991). In mammals, application of acetylcholine to cultured respiratory epithelial explants also increases ciliary activity, but the actual neuronal component has yet to be described (Corssen and Allen, 1959; Iravani and Melville, 1975). Thus, several neurotransmitters have been shown to directly control ciliary activity. However, the physiological relevance in many cases is tentative at best, as endogenous pathways containing the neurotransmitter are often not described. It is likely that in the future, several additional neurotransmitters will be shown to have cilio-regulatory actions.

VI. Intracellular Signaling Pathways Involved in Ciliary Regulation

Of the numerous cellular signaling pathways activated by 5-HT in non-ciliated systems, only cyclic AMP (cAMP) and Ca^{2+} appear to underlie the effect of 5-HT in the ciliated systems studied to date. Whereas 5-HT has been implicated in regulating ciliary activity in numerous systems, the lateral gill cilia of *Mytilus* is the only system in which a signaling pathway capable of mimicking 5-HT-regulated ciliary activity has been determined. In this system, 5-HT-induced cilio-activation is mimicked by increases in intracellular cAMP concentrations (Murakami, 1983; 1987) and is independent of alterations in membrane potential (Saimi *et al.*, 1983a). However, definitive proof that cAMP is the second messenger underlying the 5-HT response has yet to be provided. Additionally, 5-HT-induced cilio-excitation is dependent on the concentration of extracellular Ca^{2+} and is associated with a relative decrease in intracellular Ca^{2+}

concentrations within the gill epithelium (Paparo and Murphy, 1975a; 1975b). Thus, cAMP and Ca^{2+} may mediate 5-HT-induced cilio-excitation in *M. edulis*.

The signaling mechanisms underlying ciliary regulation in response to other neurotransmitters and mechanical stimuli have been investigated in numerous ciliary systems. These investigations have again revealed that the 2 main signaling pathways regulating ciliary activity are Ca^{2+} and cAMP. From protozoans to invertebrates to vertebrates, one or both of these intracellular mediators has been demonstrated to control ciliary motility. However, other signaling molecules, such as diacylglycerol / protein kinase C, arachidonic acid metabolites, nitric oxide, and cyclic GMP have also been shown to regulate ciliary beating.

i) Ca^{2+} Regulates Ciliary Activity

Ca^{2+}, a ubiquitous intracellular messenger, has been demonstrated to play a diverse set of roles in ciliary systems, including stimulation, inhibition, arrest and reversal of ciliary activity. Additionally, wherever the interaction has been investigated, the effects of Ca^{2+} are associated with the Ca^{2+}-binding protein calmodulin (CaM). The different types of ciliated epithelial cells in the gill of *M. edulis* display a diverse range of beat patterns in response to Ca^{2+}. In the lateral gill cilia, cilio-excitation and ciliary arrest are discrete physiological responses to different levels of intracellular Ca^{2+} within the gill (Paparo and Murphy, 1975a; 1975b), the result of 5-HT and DA release, respectively. Cilio-excitation results from low frequency stimulation of the branchial nerve (Paparo and Murphy, 1975a), most likely through the release of 5-HT (Paparo and Murphy, 1975b). Elemental analysis has determined that 5-HT-stimulated cilio-excitation is mediated by a decrease in intracellular Ca^{2+} in the gill epithelium, compared to control concentrations of Ca^{2+} (Paparo and Murphy, 1975b). Contrary to the effect of low frequency stimulation, high frequency stimulation of the branchial nerve inhibits ciliary

activity (Paparo and Murphy, 1975a), most likely through the release of DA (Paparo and Murphy, 1975b). Release of relatively high concentrations of Ca^{2+} within the gill are believed to underlie DA-induced cilio-inhibition (Paparo and Murphy, 1975a; 1975b). Further investigations have revealed that cilio-inhibitory stimuli, including high frequency stimulation of the branchial nerve (Saimi *et al.*, 1983a), direct electrical excitation (Saimi *et al.*, 1983b), or mechanical stimulation (Murakami and Machemer, 1982) of the lateral cells arrest cilia via a Ca^{2+}-dependent receptor potential. Since addition of the Ca^{2+} ionophore A23187 also induces ciliary arrest (Satir, 1975), these results strongly suggest that elevated concentrations of intracellular Ca^{2+} in the lateral gill epithelium results in ciliary arrest. Furthermore, the CaM antagonist trifluoperazine (TFP) reverses Ca^{2+}-dependent ciliary arrest (Reed and Satir, 1980; Stommel, 1984b), indicating that Ca^{2+}-CaM interactions underlie the ciliary arrest response.

In addition to the lateral gill cilia, regulation of activity in the abfrontal gill cilia of *M. edulis* is mediated by Ca^{2+}-dependent processes. The abfrontal cilia are quiescent until mechanically stimulated, whereupon the cilia commence beating. This cilio-activation is mediated by Ca^{2+}-dependent generator and regenerative potentials, providing an influx of Ca^{2+} possibly through voltage-dependent calcium channels (Stommel, 1984a). Addition of A23187 activates ciliary beating in the abfrontal cilia (Stommel, 1984b), whereas increased CBF upon application of Ca^{2+} is abolished by CaM inhibitors (Stommel, 1984b). Taken together, these results suggest that the cilio-excitatory response is due to increased intracellular Ca^{2+} concentration acting through CaM, in a manner similar to the ciliary arrest response observed in lateral gill cilia of *Mytilus*.

Ciliary activity in numerous other invertebrate systems is modulated by Ca^{2+} and Ca^{2+}-dependent processes. As part of the feeding behavior, macrocilia of the ctenophore *Beroë gracilis* are activated in response to sensory stimuli to assist movement of the lips

over the prey. Sensory stimuli depolarize the macrocilia causing voltage-dependent Ca^{2+} channels to open, an influx of extracellular Ca^{2+}, and subsequent cilio-excitation (Tamm, 1988). The cilio-stimulatory response is inhibited by W-7, a CaM antagonist, suggesting that Ca^{2+}-CaM interactions underlie macrocilia activation (Tamm, 1988). Likewise, an influx of extracellular Ca^{2+} underlies the adrenergic stimulation of the trochal cilia in the trochophore larvae of the polychaete *Spirobranchus giganteus* (Marsden and Hassessian, 1986). Additionally, TFP caused a transient slowing of ciliary beating, once again suggesting that the effect of Ca^{2+} on cilio-excitation may be in part mediated by CaM (Marsden and Hassessian, 1986).

In vertebrate systems, cilio-excitatory effects occur in response to a variety of stimuli, including application of exogenous neurotransmitters, mechanical stimulation, or as a reaction to factors associated with immune host-defense mechanisms. Independent of the mechanism of stimulation, Ca^{2+}-dependent effects underlie cilio-excitation in almost all systems examined. For instance, exogenous ATP application to cultured rabbit tracheal epithelium causes concurrent increases in CBF and intracellular Ca^{2+} concentration (Korngreen and Priel, 1994). Likewise, ATP-induced cilio-excitation in frog palate epithelium and esophagus is mediated by increases in intracellular Ca^{2+}. In this case though, the cilio-excitatory effect occurs secondarily to increased Ca^{2+}, as enhanced ciliary activity is via hyperpolarization of the membrane due to activation of Ca^{2+}-dependent K^+ channels (Weiss *et al.*, 1992; Tarasiuk *et al.*, 1995). Additionally, elevated intracellular Ca^{2+} concentrations increase ciliary beating in ovine tracheal epithelial cells in response to ACh application (Salathe and Bookman, 1995).

Similar to neurotransmitter regulation of ciliary activity, mechanical stimulation of cultured rabbit tracheal cilia causes cilio-excitation. Increased intracellular Ca^{2+} concentrations, through mobilization of Ca^{2+} from intracellular stores, underlies the excitatory response (Sanderson *et al.*, 1992). In this system, cells adjacent to those

receiving mechanical stimuli also undergo cilio-excitation, presumably via the movement of IP_3 through gap junctions to activate Ca^{2+} release (Sanderson *et al.*, 1992). Finally, as part of a non-specific host defense mechanism, cilio-excitatory responses in human nasal epithelial cells are mediated by increases in intracellular Ca^{2+} concentration (Di Benedetto *et al.*, 1991). Again, the cilio-excitatory effect of Ca^{2+} is abolished by the CaM antagonist TFP, further suggesting a role for Ca^{2+}-CaM interactions in modulating ciliary activity.

In general, ciliary arrest or reversal responses are also mediated by alterations in the concentration of intracellular Ca^{2+}. Ciliary inactivation (cilia in an upright, inactive posture) and arrest in stigmatal cilia of *Ciona intestinalis* are differentially influenced by increased intracellular Ca^{2+} concentrations. The arrest response has a higher threshold for increased Ca^{2+}, as inactivation and arrest occur in response to addition of A23187 in the presence of 50 mM and 100 mM $CaCl_2$, respectively (Bergles and Tamm, 1992). Ciliary reversal is also controlled by Ca^{2+}. Addition of A23187 to comb plates of the ctenophore *Mnemiopsis leidyi* causes ciliary reversal, indicating that reversal is mediated by increased intracellular Ca^{2+} concentration (Nakumara and Tamm, 1985). A similar response to Ca^{2+} is observed in ciliates, such as *Paramecium sp.* and *Didinium nasutum*. In these organisms, increased backward swimming is due to ciliary reversal, which can be initiated by a depolarization of the membrane, followed by a subsequent Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Bonini *et al.*, 1991; Pernberg and Machemer, 1995a). In *Paramecium*, these Ca^{2+} channels have been localized to the ciliary membrane (Bonini *et al.*, 1991). Consistent with most other ciliary responses examined, the regulation of ciliary reversal is likely mediated by Ca^{2+}-CaM interactions within the cilium (Nakaoka *et al.*, 1984).

It is clear that intracellular Ca^{2+} plays an essential role in regulation of ciliary activity in several species, from protozoans to invertebrates to vertebrates. In many of the

above mentioned studies, CaM antagonists inhibit the Ca^{2+}-dependent effect on ciliary activity, strongly suggesting the importance of Ca^{2+}-CaM interactions.

ii) Cyclic AMP Mediates Ciliary Activity

Another common regulator of ciliary activity is cyclic AMP (cAMP). However, as compared to Ca^{2+}, the role of cAMP as a signaling agent is not as widespread in the systems examined to date. In the lateral gill cilia of *M. edulis*, 5-HT-induced cilio-excitation can be mimicked by pharmacological agents known to increase intracellular cAMP concentrations (Murakami, 1983; 1987); this response appears to be independent of extracellular Ca^{2+} (Murakami, 1987). Even though these results suggest a role for cAMP, no inhibitors of cAMP have been tested in this system, thus definitive proof that 5-HT acts through a cAMP pathway has not been provided. Likewise, cAMP has been shown to regulate ciliary activity in stigmatal cilia of *Ciona intestinalis*. Application of cAMP produced cilio-excitatory effects, whereas addition of Ca^{2+} caused ciliary inactivation. Thus, cAMP and Ca^{2+} may have antagonistic effects (Bergles and Tamm, 1992). In *Paramecium*, swimming speed and direction are controlled by membrane potential. Hyperpolarization of the membrane increases the speed of forward swimming and this effect is mimicked by increases in intracellular cAMP (Bonini *et al.*, 1986). Although the specific interaction between hyperpolarization and cAMP is unknown, it has been postulated that hyperpolarization may activate adenylate cyclase, the enzyme responsible for production of cAMP (Bonini *et al.*, 1991).

Cyclic AMP has also been shown to modulate ciliary activity in vertebrate systems. Pharmacological activation of β-adrenergic pathways increases ciliary beat frequency (CBF) in mammalian respiratory epithelium (Verdugo *et al.*, 1980). This cilio-excitation effect can be mimicked by application of the cAMP analog, dibutyryl cAMP, as shown in experiments on human respiratory epithelium (Di Benedetto *et al.*, 1990a). In

rabbit tracheal epithelium, activation of cAMP, through addition of membrane permeable analogs or other agents known to increase intracellular cAMP, increased CBF (Tamaoki *et al.*, 1989; Lansley *et al.*, 1992). In frog palatine epithelial cells, cAMP may underlie noradrenaline-induced cilio-excitation (Maruyama, 1984). These effects of cAMP are postulated to be mediated by cyclic AMP-dependent protein kinase A (PKA) pathways. Phosphorylation events, initiated by activation of PKA, are hypothesized to regulate ciliary beating through interactions within the cilium (Di Benedetto *et al.*, 1990b; see VII). Thus, cAMP is a potent regulator of ciliary activity in many systems.

iii) Additional Signaling Pathways Controlling Ciliary Activity

Several additional signaling pathways have been shown to modulate CBF, such as nitric oxide, cyclic GMP, arachidonic acid metabolites, and diacylglycerol / protein kinase C. Activation of these pathways occurs through a variety of stimuli in numerous ciliary systems.

In an attempt to understand the intracellular pathways mediating alcohol-induced alterations in ciliary beating, ethanol was applied to bovine bronchial cells. Nitric oxide (NO)-dependent pathways were stimulated by ethanol, which appears to act through interactions with β-adrenergic receptors (Sisson, 1995). In another study, NO increased ciliary activity in previously stimulated bovine bronchial epithelial cells (Jain *et al.*, 1993) In both cases, inhibitors of nitric oxide synthase blocked the increase in CBF. However, these experiments were performed on aggregates of ciliated epithelial cells, therefore it is not known whether NO is formed within these cells or acts as a distant transmitter. NO has also been postulated to activate guanylate cyclase, the enzyme responsible for production of cyclic GMP (cGMP). Thus, the effects of NO may be mediated by cGMP, which on its own, regulates ciliary activity. Cyclic GMP increases CBF in human nasal epithelium, mimicking the effect of C-type natriuretic peptide (CNP;

Geary *et al.*, 1995). A Ca^{2+}-CaM-regulated guanylate cyclase has been identified in the ciliary membrane from *Tetrahymena* (Schultz *et al.*, 1983), but a role for cGMP in this system has not been identified. Taken together, these results suggest that cross-talk interactions between NO and cGMP may be involved in the regulation of ciliary beating in several types of ciliated epithelial cells.

Mucous transport in the respiratory tract of vertebrates is dependent on ciliary activity. The activity of the cilia can be modulated by a variety of chemical mediators released during an immune response. Arachidonic acid (AA) metabolites exemplify such mediation. Application of a stable mimetic of thromboxane A_2, a cyclooxygenase product of arachidonate metabolism, stimulates rabbit tracheal epithelial cells (Chiyotani *et al.*, 1992). This effect was significantly abolished by specific inhibitors of AA metabolites, Ca^{2+}, and PKC, which suggests that cilio-stimulation may be mediated by Ca^{2+} influx and PKC, in addition to thromboxane A_2 (Chiyotani *et al.*, 1992). CBF in respiratory cilia does not decrease under normal circumstances, but decreases are observed in individuals with bronchial asthma (Weisman *et al.*, 1990). In an attempt to identify possible cilio-inhibitory substances, the effect of another AA metabolite, leukotriene C_4, was examined. In chicken trachea, leukotriene C4 decreased CBF in a Ca^{2+}-dependent manner (Weisman *et al.*, 1990). Thus, AA metabolites are good candidates for signaling pathways involved in regulation of CBF in respiratory epithelium.

Diacylglycerol (DAG) / protein kinase C (PKC) pathways have also been implicated in control of ciliary activity. In rabbit and bovine tracheal epithelial cells, activation of PKC-dependent mechanisms through application of phorbol esters or diacylglycerol analogs causes cilio-inhibition (Kobayashi *et al.*, 1988; Salathe *et al.*, 1993). PKC inhibitors prevent cilio-inhibition, suggesting a direct role for PKC. Cilio-inhibition in these systems results from immune responses to inhaled particles, such as bacteria. In contrast, cilio-excitation upon ATP challenge in frog esophageal tissue is

dependent on PKC activation (Levin *et al.*, 1997). PKC may then activate Ca^{2+} influx through voltage-insensitive Ca^{2+} channels; Ca^{2+} is postulated to be responsible for the cilio-excitatory effects observed upon addition of ATP. Taken together, these studies demonstrate that numerous signaling pathways have evolved to modulate ciliary activity in eukaryotic systems. In any one system, interactions between a number of signaling pathways is likely to be important in regulation of ciliary activity.

VII. Intraciliary Regulation of Ciliary Beating

In the studies described above, experiments were performed on isolated tissue pieces or single ciliated cells. However, as ciliary activity is highly regulated, it is likely that rapid small-scale changes in signaling pathways occur within the cilium. In order to address this hypothesis, many experimenters have taken advantage of cell models of cilia. Cell models are composed of cilia isolated from the cell and detergent-permeabilized, permitting direct access to the axoneme, the main component of the cilium. Results from experiments with cell models have provided an abundance of information regarding the regulation of ciliary beating by signaling agents such as cAMP, Ca^{2+}, CaM, and PKC.

Within the cilium, Ca^{2+} has been shown to be a ubiquitous signaling messenger, similar to the cilio-regulatory effects of Ca^{2+} witnessed in whole ciliated cell experiments (see section VI-i). The sliding microtubule model of ciliary beating suggests that doublet microtubules slide relative to one another due to the activity of the bridged dynein arms (see section II). Experiments on ciliary doublet microtubules from *Tetrahymena* have shown that the doublets glide along dynein-coated glass surfaces in a Ca^{2+}-dependent manner, implicating Ca^{2+} in regulation of microtubule sliding within the axoneme (Mori and Miki-Noumura, 1992). Similar axonemal interactions with Ca^{2+} are seen in dissociated macrociliary cells of the ctenophore *Beroë*, where stimulation of the ciliary beat requires the presence of Ca^{2+} (Tamm, 1988). Likewise, in another ctenophore,

Mnemiopsis leidyi, exposure of ATP-reactivated models of comb plates to Ca^{2+} causes ciliary reversal (Nakumara and Tamm, 1985). Taken together, these results strongly implicate Ca^{2+} as a modulator of ciliary activity within the axoneme. Further evidence implicating a direct role for Ca^{2+} at the axoneme comes from the visualization of Ca^{2+} transients within the cilium. Using Ca-Green dextran-injected larvae of *M. leidyi*, Tamm and Terasaki (1994) were able to visualize fluorescence within the cilium, which corresponded to specific levels of Ca^{2+}. Changes in fluorescence occurred along the entire length of the cilium when stimulated. Analysis of the mechanics and time-dependency of the transients revealed that the changes in fluorescence could not be due to diffusion of Ca^{2+}, thus the results suggest that voltage-dependent Ca^{2+} channels are distributed over the length of the cilium (Tamm and Terasaki, 1994). Contrary to these findings are the results obtained from detergent-permeabilized cells from *Paramecium*. Although ciliary reversal is mediated by interactions with Ca^{2+} (Bonini *et al.*, 1991), addition of Ca^{2+} to the base of the cilium resulted in the greatest degree of reversal, suggesting that the sites of Ca^{2+} entry are located at the base of the cilium and not along the entire length of the cilium, as observed in *M. leidyi*. However, independent of the site of entry, Ca^{2+} plays a major role in regulation of ciliary beating at the level of the axoneme.

Ca^{2+}-dependent regulation of ciliary activity has been demonstrated in the bivalve *M. edulis*. Stimulation of the lateral gill cilia causes a transient ciliary arrest through Ca^{2+}-dependent pathways in isolated gill tissue pieces (see section V). A transient ciliary arrest was also observed upon addition of Ca^{2+} to permeabilized lateral gill cilia, suggesting a direct role for Ca^{2+} at the level of the axoneme (Stommel and Stephens, 1985a). A detailed examination of phosphorylation within arrested lateral gill cilia has demonstrated the presence of Ca^{2+}-dependent phosphorylation of lipids; *i.e.* bis-phosphoinositols (PIP_2) formed through the phosphorylation of PIP (Stommel and

Stephens, 1985a). The transient nature of the arrest response is postulated to involve PIP_2, which results in activation of Ca^{2+}-ATPases, lower internal Ca^{2+} concentrations, and resumption of beating. Therefore, in addition to direct effects of Ca^{2+} within the axoneme, Ca^{2+}-dependent phosphorylation events are also likely involved in the complex regulation of ciliary activity. Moreover, one or several Ca^{2+}-binding molecules must be present within the axoneme to account for the numerous roles played by Ca^{2+}.

Calmodulin (CaM), a ubiquitous Ca^{2+}-binding protein, has been suggested to underlie a significant portion of Ca^{2+}-dependent events in whole ciliated cell studies (see section VI-i). Regulation of ciliary beating through CaM has been demonstrated in cell models in numerous systems, including *Tetrahymena* (Ohnishi *et al.*, 1982), *Aequipecten irradians*, (Stommel *et al.*, 1982), *Ciona intestinalis* (Bergles and Tamm, 1992), and *M. edulis* (Stommel and Stephens, 1985b). Several mechanisms for the action of CaM within the axoneme have been proposed, including mediating Ca^{2+}-dependent effects on dynein-microtubule sliding, modulation of Ca^{2+}-ATPase transport systems, and buffering of Ca^{2+} within the cilium (Stommel *et al.*, 1982). However, the localization of CaM within the axoneme varies from organism to organism. In *Tetrahymena*, CaM is found along the longitudinal axis of the outer doublet microtubules. It repeats every 90 nm and is possibly associated with the interdoublet (nexin) links (Ohnishi *et al.*, 1982). Likewise, CaM was localized to the axoneme of lateral gill cilia in bivalves, but no CaM binding was present in dynein extracts (Stommel and Stephens, 1985b). Contrary to these findings, CaM was found within the cilium of scallop gill epithelial cells, but was not bound to the axoneme proper (Stommel *et al.*, 1982). Thus, the Ca^{2+}-dependent arrest responses observed in *Tetrahymena*, *Mytilus*, and *Ciona* appear to be mediated by CaM. However, the specific localization of CaM within the cilium mediating the arrest response is not a general phenomenon.

Cyclic AMP has also been shown to regulate ciliary activity at the level of the axoneme. In many cases, the effects of Ca^{2+} and cAMP are opposite; one is usually able to modulate the activity of the other. Therefore, Ca^{2+} and cAMP may be involved in the interaction between the active, cycling state versus the inactive, non-cycling states of ciliary beating, possibly through modulation of the timing or pattern of dynein-microtubule interactions (Sanderson *et al.*, 1992).

Opposite roles of Ca^{2+} and cAMP have been demonstrated in ATP-reactivated cell models of cilia from *Ciona intestinalis* and *M. edulis*. Cyclic AMP stimulates beating in *C. intestinalis*, whereas increased Ca^{2+} concentration causes cilia to assume an inactive position (Bergles and Tamm, 1992). Likewise, in ATP-treated permeabilized lateral gill cilia of *M. edulis*, cAMP re-activates ciliary activity in Ca^{2+}-arrested cilia (Stommel and Stephens, 1985b), providing further evidence for opposing roles of cAMP and Ca^{2+} at the level of the axoneme. In cell models of *Paramecium*, interactions between Ca^{2+} and cAMP have also been observed. The activity of adenylate cyclase, and thus the level of cAMP, is modulated by Ca^{2+} entering the cilium through voltage-sensitive Ca^{2+} channels (Gustin and Nelson, 1987).

The effects of Ca^{2+} and cAMP on ciliary activity are unlikely to occur through direct binding of the signaling agent to the axoneme. A more likely explanation is the activation of subsequent Ca^{2+}- and cAMP-dependent events, such as phosphorylation or interactions with binding proteins, such as CaM. Phosphorylation of axonemal proteins by protein kinase A may mediate the effects of cAMP observed in permeabilized cell models. For instance, axokinin, the regulatory subunit of type 2 PKA, is a 56 kDa soluble PKA substrate phosphorylated in a cAMP-dependent manner upon activation of motility (Tash, 1989). Effects of Ca^{2+} are likely the result of Ca^{2+}-CaM interactions. Such interactions may initiate phosphatase activity, resulting in the dephosphorylation of PKA-phosphorylated axonemal proteins. Thus, phosphatase activity could counteract the effect

of PKA-dependent phosphorylation. In contrast to phosphorylation-dephosphorylation events, the specific effects of Ca^{2+} and cAMP may be due to modulation of separate axonemal proteins. For instance, PKA may phosphorylate a specific subset of dynein-associated regulatory proteins, whereas Ca^{2+}-CaM kinases may phosphorylate a separate subset of proteins (Stommel and Stephens, 1985b). Thus, the opposing effects of Ca^{2+} and cAMP may be due to opposite effects on a single axonemal component or through separate effects on different axonemal components.

In order to fully understand the control of ciliary activity, both whole cell and intraciliary approaches are necessary. Evidence for this comes from the results of whole cell and permeabilized cell studies of rabbit tracheal epithelial cells. Using whole cells, significant ciliary activity in response to Ca^{2+} and cAMP was observed. However, using permeabilized cells, no effect of cAMP or Ca^{2+} on ciliary beating was evident (Lansley *et al.*, 1992). The authors suggest that the permeabilization process may remove the components with which Ca^{2+} and cAMP normally interact. Conversely, cAMP and Ca^{2+} may act within the cell body, regulating the availability or the metabolism of ATP, and in this manner affect ciliary activity indirectly (Lansley *et al.*, 1992). Support for interactions between Ca^{2+} and ATP is found in an examination of ciliated epithelial cells in the oviduct of *Necturus maculosus*. In whole cells, ciliary activity was dependent on Ca^{2+} influx (Murakami and Eckert, 1972), whereas in ATP-reactivated cell models, activity was directly dependent on the concentration of energy source available (ATP). Thus, the effect of Ca^{2+} in whole cells was postulated to be an indirect effect on activity, possibly acting as a rate-limiting step in the metabolic pathways leading to ATP synthesis (Eckert and Murakami, 1972).

The number of signaling pathways that have been implicated in the regulation of ciliary activity in whole cells is vast. Similarly, the control of ciliary activity by signaling agents at the level of the axoneme is also likely to be a very complex process. In addition

to the effects of Ca^{2+} and cAMP, PKC and cyclic GMP-dependent protein kinase G (PKG) have been found to modulate ciliary activity in permeabilized cell models. In sheep tracheal epithelium, phosphorylation of a 37 kDa protein by the specific action of PKC results in a significant decrease in CBF (Salathe *et al.*, 1993). PKG is hypothesized to phosphorylate ciliary proteins within the *Paramecium* axoneme to regulate orientation of the effective stroke (Ann and Nelson, 1995). Thus, the control of ciliary beating at the level of the axoneme appears to be a complex series of interactions, mediated by a variety of signaling pathways. In addition, permeabilized cell studies are an important complement to whole ciliated cell studies in order to obtain a detailed and accurate view of the control of ciliary beating.

VIII. Ciliated Cells in Embryos of *Helisoma trivolvis*

Embryos of the pulmonate gastropod *Helisoma trivolvis* develop within a gelatinous egg mass that contains numerous spherical egg capsules, each housing an individual animal. The transparent nature of the egg mass permits the observation of behavioral responses of embryos throughout development. During early stages of development, embryos display a rotation behavior driven by the coordinated beating of 3 bands of ciliated epithelium, 2 dorsolateral prototrochal bands and a ventromedial pedal band (Diefenbach *et al.*, 1991). This rotation behavior is composed of two components, a slow, basal rate of rotation and accelerated bouts, or surges, of rotation caused by increased ciliary activity. This behavior first appears concurrently with the development of the first pair of serotonergic neurons, embryonic neurons C1 (ENC1; Diefenbach *et al.*, 1991). Previous pharmacological and immunohistochemical studies have revealed that the ventromedial band of cilia is innervated by ENC1 (Diefenbach *et al.*, 1991; Goldberg *et al.*, 1994; Diefenbach and Goldberg, 1995; Goldberg *et al.*, 1996). The release of 5-HT from ENC1 onto the ciliated epithelium is believed to produce the periods of increased

ciliary activity underlying rotational surges. Furthermore, each ENC1 contains an apical dendrite that projects to the embryo surface and is tipped with a chemosensory-like structure (Goldberg *et al.*, 1996). These findings have prompted the hypothesis that ENC1 is a sensory-motor neuron that modulates cilia-driven rotation in response to changes in the extra-embryonic environment.

Pharmacological examination of the 5-HT receptor underlying the cilio-excitatory response suggests the involvement of a 5-HT receptor with a novel pharmacological profile (Goldberg *et al.*, 1994). The agonist-antagonist activation properties of this 5-HT receptor share properties with both the 5-HT_1 and 5-$HT_{2/1C}$ receptor subtypes described in vertebrate systems (Goldberg *et al.*, 1994). The 5-HT_1 receptor subtype is a family of G-protein coupled receptors that classically works through a G_i-protein, which is negatively linked to adenylate cyclase (Hen, 1992). In contrast, the 5-$HT_{2/1C}$ receptor subtype is linked via an unknown G-protein to phospholipase C activation, resulting in the formation of the intracellular signaling agents, IP_3 and DAG (Hen, 1992). Despite the partial similarity with vertebrate receptors, it is likely that the *Helisoma* ciliary receptor does not belong specifically to one of these families. Molecular analyses of cloned 5-HT receptors from *Lymnaea stagnalis* (Sugamori *et al.*, 1993) and *Aplysia californica* (Li *et al.*, 1995) did not reveal a close structural relationship with any specific vertebrate receptor. However, these and other molluscan receptors (Price and Goldberg, 1993a; 1993b) do interact with the cAMP and IP_3/DAG second messenger systems, as do the vertebrate subtypes. Taken together, this pharmacological evidence suggests the cAMP and IP_3/DAG/PKC signaling systems are likely candidates to mediate the cilio-excitatory action of the *Helisoma* ciliary 5-HT receptor.

A majority of the past research on ciliary systems suffers from the shortcoming that information on neural control, intracellular regulation, and overall biological function has not been well-integrated in any one system. In the lateral gill cilia of *M. edulis*, an

effort has been made to examine the signaling pathways activated by 5-HT. However, direct evidence for a role of cAMP or Ca^{2+} has not been produced, as only mimetic effects have been observed. The ability of a signaling agent to mimic the effect of a neurotransmitter does not necessarily mean that the neurotransmitter works through that particular pathway. Only when a strategy is used to block endogenous pathways can the direct role of a signaling agent be proposed to be involved.

The work to date on *Helisoma* embryos suggests that they are an excellent model system for implementing an integrated approach in studying ciliary control. ENC1s, the serotonergic neurons that innervate the pedal ciliated cells, have been extensively studied in terms of their development (Diefenbach, 1995), innervation and control of pedal ciliated cells (Goldberg *et al.*, 1996; Diefenbach *et al.*, 1991), transmitter properties (Goldberg and Kater, 1989; Diefenbach and Goldberg, 1995; Cole, Young, and Goldberg, unpublished observations), and biological role as sensory-motor cilio-excitatory neurons (Diefenbach *et al.*, 1991; Goldberg *et al.*, 1996). The ciliated cells themselves have been subjected to both *in vivo* and cell culture analyses, providing information on the pharmacological properties of the ciliary 5-HT receptors (Goldberg *et al.*, 1994), their possible signaling molecules (Cole and Goldberg, unpublished), and their role in mediating the embryonic rotation behavior (Diefenbach *et al.*, 1991; Diefenbach, 1995). These advantages, taken together with the ability to isolate ciliated cells in culture and reliably and efficiently measure their ciliary beat frequency, make this system ideal to investigate the intracellular signaling pathways that mediate the ciliary response to the naturally occurring neurotransmitter, 5-HT. Moreover, it provides a unique opportunity to gain a truly integrated understanding of a neurally-controlled ciliary system.

The overall goal of this thesis is to understand the intracellular signaling pathways involved in 5-HT-induced cilio-excitation. Using primary cell cultures of dissociated early *Helisoma* embryos, the direct effect of the neurotransmitter 5-HT on ciliary activity

can be examined. Time-lapse videomicroscopy facilitates the resolution of single ciliary beats, thus allowing for a direct examination of how various agents affect ciliary activity. Through pharmacological manipulations, the effect of signaling pathways on CBF can be determined. Additionally, measurements of intracellular Ca^{2+} within isolated ciliated cells can be obtained through experiments involving the Ca^{2+}-sensitive fluorescent indicator, Fura-2. The main objectives of this thesis were to:

(1) characterize the direct effects of 5-HT on ciliary activity of cultured ciliated cells

(2) test the roles of the cAMP, Ca^{2+}, and protein kinase C (PKC) signal transduction pathways in 5-HT-stimulated ciliary activity

(3) establish techniques to measure levels of intracellular Ca^{2+} in ciliated cells.

Evidence is put forth that 5-HT directly activates ciliary activity in cultured ciliated cells via Ca^{2+}- and PKC-dependent pathways, whereas cAMP is not involved in modulating ciliary activity in this system.

MATERIALS AND METHODS

I. Animals and Drugs

An inbred, laboratory-reared albino colony of *Helisoma trivolvis* was raised in filtered, flow-through glass aquaria (45 l) with an oyster shell substratum (Diefenbach and Goldberg, 1990). The snails were maintained at 25°C on a 12:12 h light:dark cycle. Their diet consisted of Trout Ration (NU-WAY-United Feeds, Calgary, AB) and romaine lettuce. Embryos are contained within individual egg capsules housed in a single egg

mass. Egg masses, containing approximately 25 sibling embryos (range 5-60; Goldberg *et al.*, 1988), were collected from the glass walls of the aquaria or from petri dishes placed at the bottom of the tanks. Egg masses were removed with a razor blade and transferred to Multiwell tissue culture plates (6-well; Falcon 3046) containing artificial pond water (APW; 0.025% Instant Ocean; Aquarium Systems, Mentor, OH). Embryos were viewed through a dissection microscope and staged for their extent of embryonic development according to the system of McKenney and Goldberg (1989). All embryos used were of embryonic stages E25-E30, which represents 25-30% of intracapsular developmental time.

Serotonin (5-HT creatine sulfate, Sigma Chemical Company, St. Louis, MO), verapamil (Sigma), nifedipine (Sigma), potassium chloride (KCl; BDH Chemicals, Toronto, ON), sodium chloride (NaCl; BDH), and 8-bromo-cyclic 3',5'-adenosine monophosphate (8-Br-cAMP; Sigma) were dissolved in *Helisoma* saline (HS: 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM HEPES; pH 7.3-7.35). Forskolin, bisindolylmalemide, Calphostin C, *sn*-1,2-dioctanoylglycerol (DiC_8), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), and *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9; all from Calbiochem, La Jolla, CA), A23187 and phorbol 12β, 13α-dibenzoate (PDBn; both from Sigma), and phorbol 12-myristate 13-acetate (TPA; Research Biochemicals International, Natick, MA) were dissolved in dimethyl sulfoxide (DMSO; BDH). Thapsigargin and veratridine (both from Research Biochemicals International) and BayK 8644 (Calbiochem) were dissolved in 100% ethanol, while 3-isobutyl-1-methylxanthine (IBMX; Sigma) was dissolved in equal amounts of 100% ethanol and HS. Experiments with Calphostin C were carried out under normal overhead fluorescent illumination, in combination with transmitted microscope light, to account for the light-dependent activation properties of this compound (Bruns *et al.*, 1991). DiC_8, OAG, and SC-9 were dissolved in 2 µl HS immediately prior to addition to the culture dishes.

II. Isolation and Culture of Ciliated Cells

Embryonic ciliated cells were cultured as described previously (Goldberg *et al.*, 1994). Intact egg masses were rinsed in 35% ethanol for 2 min, followed by 1 x 2 min and 2 x 10 min washes in antibiotic-containing *Helisoma* saline (AHS: HS +150 µg per ml gentamycin; Sigma). Embryos were isolated from their egg capsules and transferred to 0.2% trypsin (type III from bovine pancreas; Sigma) in AHS for 20-45 min. Embryos were transferred to 0.1% trypsin inhibitor (type I-S from soybean; Sigma) in AHS for 15 min and subsequently washed in *Helisoma*-defined medium (HDM: 50% Liebovitz-15 (Gibco, Grand Island, NY), 40.0 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM HEPES, 50 µg per ml gentamycin, 0.015% L-glutamine; pH 7.3-7.35) for 10 min. The embryos were mechanically dissociated by passing them repeatedly through a 63 µm nylon mesh (Small Parts Inc., Miami, FL) attached to the tip of a 3 ml syringe. For time-lapse videomicroscopy experiments, the cell suspension was plated in 0.5 ml droplets onto poly-L-lysine-coated (hydrobromide; MW 4000-15000; 1 mg/ml; Sigma) plastic 35 mm tissue culture dishes (Falcon 3001) that had been inscribed with circles to contain the droplet. For calcium imaging experiments, the cell suspension was plated in 0.5 ml aliquots into 35 mm poly-L-lysine-coated glass-bottom (number 1 coverslip) petri dishes. The plates were covered, humidified, and kept at room temperature overnight (20-24 h) to allow for cell adhesion. The following day, one of two protocols was employed: (1) 1.5 ml of HDM was added to the culture dishes; or (2) 0.5 ml of HDM was removed and replaced with 2 ml of *Helisoma* saline supplemented with 5 mM dextrose (HSD). A minimum 60 min incubation in HSD was required before use in experiments to allow ciliated cells to resume active beating (see Results section VI-i). The cultured cells were used for experiments on this same day.

For experiments involving Ca^{2+}-depleted defined medium (50% Liebovitz-15, 40.0 mM NaCl, 1.7 mM KCl, 5.6 mM $MgCl_2$, 5.0 mM HEPES, 1.0 mM EGTA, 50 µg per ml gentamycin, 0.015% L-glutamine; pH 7.3-7.35), the following procedure was employed. On the day after plating, the defined medium was replaced with 2.0 ml of either "normal Ca^{2+} medium" or Ca^{2+}-depleted medium.

III. Identification of Ciliated Cells in Culture

The *Helisoma* embryonic cell culture contains a variety of isolated cell types, including neurons, myocytes and ciliated cells (Goldberg *et al.*, 1988). Of the latter, various easily distinguishable subtypes were observed. Flame cells derived from the protonephridia had a unique morphology, with a few long cilia that beat rapidly in a very distinct waveform. As these cells are not involved in the embryonic locomotory behavior, they were not examined in this study. In contrast, the remaining ciliated cells were usually large (diameter > 20 µm), round, and had a distinct dense granular appearance under phase-contrast or Nomarski differential-interference-contrast (DIC) optics (Figure 3). Furthermore, they were bordered by actively beating cilia that were often restricted to incomplete portions of the cell margin. Most of these cells had rather long cilia, similar to those seen on the pedal and prototrochal ciliary bands seen in a scanning electron microscope analysis of *Helisoma* embryos (McKenney and Goldberg, 1989). A smaller number of ciliated cells had distinctly shorter cilia that were difficult to analyze in the time-lapse CBF assay. These latter cells, which may be derived from the ciliated gut epithelium, were generally not recorded from in the present study.

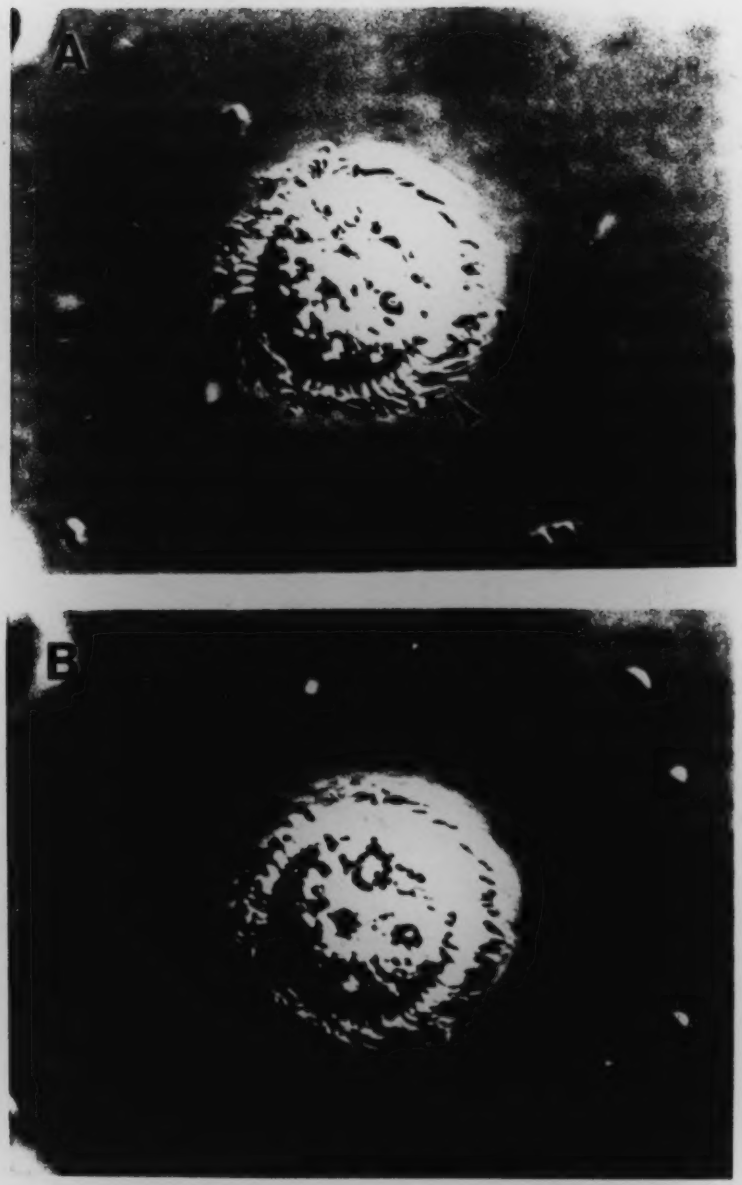


Figure 3: Nomarski differential-interference-contrast (DIC) micrographs of an isolated ciliated cell in culture. Two focal planes are shown (A and B). Individual cilia (arrowhead) and nucleolus (*) are clearly seen. Scale bar = 11 μm.

IV. Time-Lapse Videomicroscopy

i) Experimental Protocol

In experiments using the bolus drug application protocol, 3-6 cells were mapped out in each culture dish on an X-Y array and their activity recorded for 10 sec on videotape. Concentrated solutions were added to cultures in volumes ranging from 2-40 µl in order to produce the desired final concentration upon dilution with the 2 ml of culture medium. The dish was gently agitated for 30 sec to facilitate complete mixing of the drug throughout the culture dish. At a specified time post-addition, the ciliary activity of identified cells was recorded for another 5 - 10 sec on videotape for later analysis (see section IV-ii). Complementary culture dishes were used to examine the effects of each dissolving solution (vehicle controls) in the absence of drug. The maximum concentration of DMSO or EtOH used in all experiments was 0.1%; this concentration of vehicle had no effect on CBF. Data were expressed as a percentage of the basal beat frequency observed prior to vehicle or drug application. The measurements taken 10 min post-treatment were used for analysis in all experiments (Diefenbach *et al.*, 1991; Goldberg *et al.*, 1994), except in those examining the time-dependent effects of drugs. In all experiments, including those testing the concentration-dependent effects of 5-HT, all quantifiable cells were included in the analysis. This is in contrast to the method of Goldberg *et al.* (1994), where only cells that demonstrated robust responses to 5-HT were included. Approximately 40% of the ciliated cells used in the 5-HT concentration-response experiments did not respond to 100 µM 5-HT. A response is defined as an increase to 120% of the basal CBF upon addition of 100 µM 5-HT.

An examination of the temporal effects of 5-HT on ciliary activity was done using a gravity-driven perfusion system, enabling rapid addition and removal of solutions to the culture dish. The inflow and vacuum were placed in close proximity to the experimental cell. The cell was perifused with HSD for 30 sec, followed with perifusion of 5-HT for 60

sec, and a 60 sec washout period with HSD. The duration of the experiment, including 30 sec of recording of cells in HDM prior to the onset of the perifusion, was recorded on video tape for later analysis (see section IV-ii). The experimental values were expressed as a percentage of the pre-perifusion value (average of 3 CBF values of cells in HDM taken every 10 sec prior to the beginning of the perifusion). Only cells with a beat frequency greater than 110% at 60 sec post-5-HT addition were used in the analysis.

ii) Data Analysis

Ciliated cells, identified by their rapidly beating cilia, were viewed through phase contrast optics on an inverted microscope (Nikon Diaphot TMD) that was connected to a CCD video camera (JVC model TK-860U). The output of the camera was fed to a time-lapse video cassette recorder (Panasonic model AG-6720), which enabled the analysis of rapidly beating cilia at a reduced playback speed, and ciliary beating was recorded on videotape for later analysis. Off-line analysis consisted of slowing the playback speed down to 1/24 normal and recording the number of ciliary beats in a one minute interval. In most cases, the number of beats was resolved to one-half of a beat cycle. In selected experiments, ciliary beating was analyzed at different playback speeds to ensure that cilia beat frequency (CBF) was not underestimated due to frequency-matching errors.

Statistical significance was determined by Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test. In each graph, error bars represent the standard error of the mean (S.E.M.).

V. Single-Cell Calcium Imaging with Fura-2

i) Quantification of Intracellular Ca^{2+} Concentrations

Concentrations of free intracellular Ca^{2+} were quantified with the use of the Ca^{2+}-sensitive fluorescent dye, Fura-2 (Grynkiewicz *et al.*, 1985). As Fura-2 is a dual-

wavelength ratiometric dye (Kao, 1994), a ratio approach was used to quantify intracellular Ca^{2+} concentrations. Fluorescence intensity measurements were made by exciting the loaded ciliated cells with 2 wavelengths of light, 340 nm and 380 nm. The ratio of the emission signals at 510 nm elicited by the 2 wavelengths was used to calculate Ca^{2+} concentrations using the equation:

$$[Ca^{2+}] = K_d \times (F_o/F_s) \times [(R-R_{min}) / (R_{max}-R)];$$

where R = ratio of the 340/380 emission signals at the point being measured; R_{min} = the minimum ratio of signals produced in a saturating EGTA (ethylene glycol-bis (b-aminoethyl Ether) N,N,N',N'-Tetraacetic acid) solution; R_{max} = the maximum ratio of signals produced in saturating Ca^{2+} solution; K_d = the dissociation equilibrium constant of Fura-2 for Ca^{2+}; and F_o/F_s = the fluorescence ratio obtained at low and saturating Ca^{2+} concentrations at 380 nm excitation (Kao, 1994).

ii) Calibration of Fura-2

The values of R_{max}, R_{min}, F_o/F_s, and K_d required for the $[Ca^{2+}]$ equation are specific for each Fura-2 recording setup (Dr. P. Guthrie, personal communication). To determine these values, 11 solutions of various Ca^{2+} concentrations (from 0 μM to 39.8 μM Ca^{2+}; Calcium calibration buffer kit with magnesium II; Molecular Probes, Eugene, OR), each containing the free acid form of Fura-2 (100 μM), were tested using a FLUAR 100X/1.3 NA oil immersion objective (Zeiss Canada, Don Mills, ON). Additionally, 2 of the above Ca^{2+} solutions (0 μM and 39.8 μM) were tested in the absence of Fura-2 to control for background fluorescence and inherent autofluorescence of the solutions. Calibration tubes (20 nm in width; In Vitro Dynamics, Rockaway, NJ) were broken into 1.5 cm lengths and filled with one of the Ca^{2+} solutions by capillary action. All 13 tubes were mounted on a modified 20x60 mm number 1 coverslip and the coverslip was placed on the microscope stage with the focal plane in the middle of each calibration tube (*i.e.*

within the solution). Each tube was systematically excited at 340 nm and 380 nm and the fluorescence intensity values recorded. Using the ALLFIT program (DeLean *et al.*, 1978; kindly provided by Dr. J.P. Chang), the following calibration parameters were calculated; $F_o/F_s = 1.95$; $R_{max} = 3.72$; $R_{min} = 0.7$. A value for K_d was determined as the half-maximal value of a plot of the ratio values for each solution against the logarithm of the Ca^{2+} concentration in the solution; $K_d = 264.5$ nM. This value for K_d is relatively close to the reported value of 224 - 225 nM, which has been determined in other calcium imaging systems (Goldberg *et al.*, 1992; Connor, 1986; Rehder and Kater, 1992).

iii) Loading Ciliated Cells with Fura-2 and Calcium Imaging Apparatus

A variety of protocols were tested to obtain maximal cytoplasmic accumulation of Fura-2, with minimal compartmentalization in intracellular organelles. A range of concentrations (2 - 20 μM) of the membrane-permeant form of Fura-2 (Fura-2 acetoxy-methyl ester; Fura-2-AM; Molecular Probes) dissolved in DMSO or 20% Pluronic F-127 (Molecular Probes) in DMSO were added to the cultured cells for a range of time intervals (30 min - 4 h) at either room temperature (~22°C) or 28°C (95% O_2, 5% CO_2), followed by repeated rinses of the cells with either HS or HSD (0 - 120 min).

Optimal loading of ciliated cells with Fura-2 was obtained with the following protocol. Ciliated cells were washed with HSD for 60 min, loaded with 10 μM Fura-2-AM for 60 min at 28°C, rinsed 3 times with HSD, and used for Fura-2 experiments a minimum of 60 min after the final rinse.

Fura-2 loaded ciliated cells were viewed on an inverted microscope (Zeiss Axiovert 135) equipped for Nomarski DIC and fluorescence microscopy. Fluorescence images were captured by an ICCD (intensified closed-coupled device; Paultek Imaging, Grass Valley, CA) video camera. Images viewed through the camera were sent to a computer (Macintosh Quadra 950) and digitized by a QuickCapture frame grabber board

(Data Translation, Mississauga, ON). Pairs of images were captured using a computer-driven filter wheel (Empic Imaging Inc., Mississauga, ON) rotating between 340 nm (filter #340HT15) and 380 nm (filter #380HT15) excitation interference filters (Omega Optical, Brattleboro, VT). Cells were exposed for 200 msec at each excitation wavelength. A neutral density filter (0.3 or 1.0; Omega Optical) was inserted into the excitation pathway to minimize bleaching of the Fura-2 signal and saturation of the camera (Goldberg *et al.*, 1992; Williams and Fay, 1990). Emitted fluorescence passed through a 430 nm dichroic mirror (#430DCLP02; Omega Optical) and a 510 nm (filter #510WB40; Omega Optical) emission filter and was transmitted to the video camera.

iv) Measurements of Free Intracellular Ca^{2+}

Fura-2-loaded ciliated cells had to meet a number of criteria before use in calcium imaging experiments. The cell must have actively beating cilia, be isolated from other cells (*i.e.* not part of a cluster of cells), and be loaded uniformally with Fura-2, as measured by fluorescence intensity. The fluorescence intensity of a ciliated cell must be greater than 60 (measured on a pixel intensity scale of 1-256) when excited with the 340 nm excitation filter through a 0.3 neutral density filter. The computer program used to quantify Fura-2 fluorescence (RATIO) accurately calculates intracellular Ca^{2+} concentration only when the fluorescence intensity of a cell is relatively high (> 60). Cells meeting all 3 criteria were used in subsequent experiments.

Basal intracellular Ca^{2+} concentration was measured in Fura-2-loaded ciliated cells in 4 areas of the cell: whole cell body, center of the cell, ciliated membrane region, and non-ciliated membrane region. Measurements were made every 5 sec for a total of 60 sec. Calculation of Ca^{2+} concentration was performed using either the computer program RATIO or NIH Image, correcting for background fluorescence in both cases. The determined value of Ca^{2+} using either program gave similar results. However, since

RATIO calculates Ca^{2+} concentration on a pixel-by-pixel basis, rather than using spatially averaged fluorescence intensity measurements, RATIO measurements of Ca^{2+} concentration were presented.

RESULTS

I. Basal Ciliary Activity

Early embryos of *Helisoma trivolvis* display a characteristic rotation behavior mediated by 3 bands of ciliated epithelial cells (Diefenbach *et al.*, 1991). The release of 5-HT from embryonic neurons C1 (ENC1) onto the ventromedial pedal band of ciliated cells is postulated to result in a transient increase in rotation (Diefenbach *et al.*, 1991). An important complement to whole-embryo behavior studies is the analysis of the response of embryonic ciliated cells in primary cell culture to 5-HT. As a first step in validating the use of cell culture as a direct method of examining ciliary activity in response to 5-HT, a comparative analysis of basal ciliary activity in intact whole embryos and in cell culture was performed.

Early embryos of *Helisoma trivolvis* possess 3 bands of ciliated epithelial cells, a ventromedial pedal band and 2 dorsolateral prototrochal bands (Diefenbach, 1995). In intact embryos, CBF in the ventromedial band ranged from 4.8 to 7.2 beats per second (n=3). Likewise, dorsolateral cells beat at frequencies ranging from 4.8 to 6.8 beats per second (n=3).[1] In primary cell culture, ciliated cells beat at a slightly faster rate, with a mean value of 8.4 ± 0.1 beats per second (N=767). Moreover, analysis of the distribution of beat frequency in the population of cultured ciliated cells revealed a normal distribution around a single peak value (Figure 4). Therefore, the cilia examined in this study behaved as a homogenous population, at least in terms of basal activity. Temporal

examination of individual ciliated cells in culture demonstrated that basal beat frequency remained stable over time when measured every 5 sec for 60 sec (Goldberg and Neumann, unpublished results). No surges or oscillations in CBF were observed, suggesting that rotational surges in whole embryos results from neuronal stimuli, rather than inherent oscillatory beating of ciliated cells.

Ciliary activity is dependent on ATP (see Introduction section II). The amount of ATP formed by the cell through glycolysis is a function of the amount of readily available sugar. To determine whether the normal incubation medium contains sufficient sugar to sustain maximal basal ciliary activity, additional dextrose was added to HDM (Dextrose - HDM; total sugar content = 20 mM). Basal CBF did not differ in ciliated cells incubated in HDM (total sugar content = 5 mM) or in Dextrose - HDM (Figure 5; $p>0.05$).[2] Thus, the concentration of sugar available in HDM is adequate to sustain maximal ciliary activity. Moreover, a minimum amount of sugar in HDM was necessary, since ciliary beating did not occur in solutions devoid of sugar (see section VI-i).

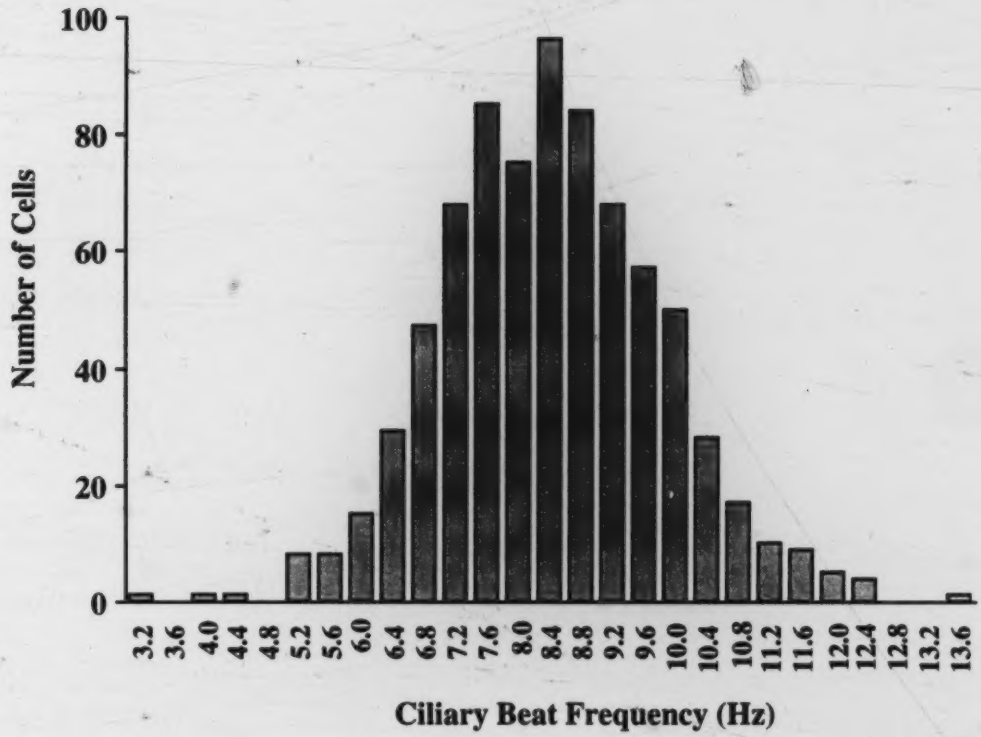


Figure 4: Distribution of basal ciliary beat frequency in the population of cultured ciliated cells. The average CBF was 8.4 ± 0.1 beats per second (N = 767 cells).

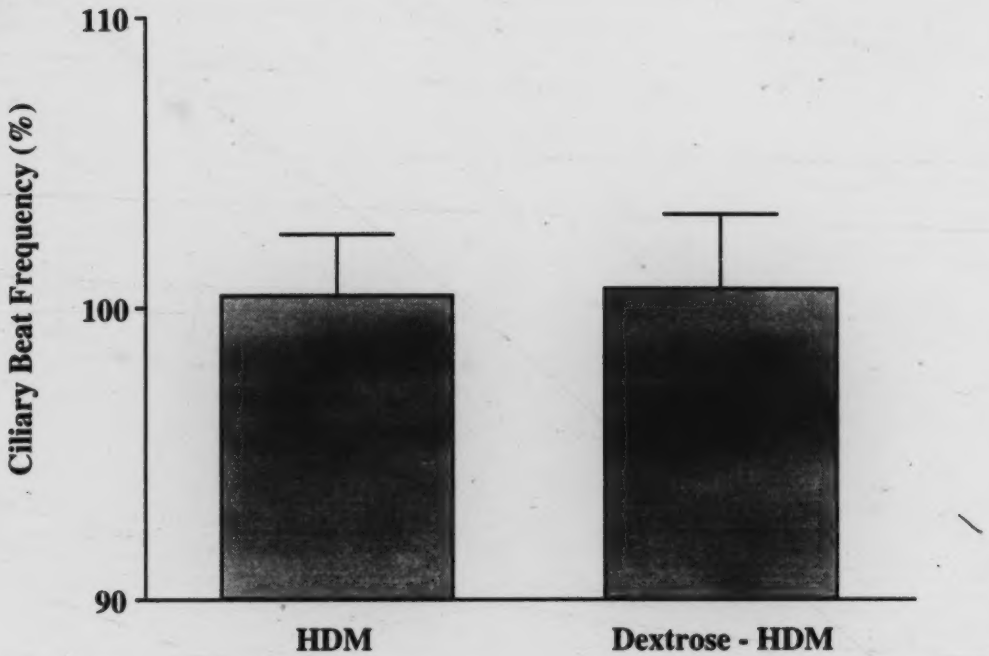


Figure 5: Additional dextrose in the bathing medium did not affect basal CBF. Values are normalized to CBF values recorded in defined medium (HDM; total sugar content is 5 mM), prior to exchange of bathing solution to either HDM or HDM with additional dextrose added (Dextrose - HDM; total sugar content is 20 mM). CBF values were obtained following incubation in either medium for 30 min. Each bar represents the average response of 31 to 33 cells.

II. Effects of Exogenous 5-HT on Ciliary Beat Frequency

5-HT is a common regulator of ciliary activity in many molluscan systems (Murakami, 1983; Syed and Winlow, 1989; Audesirk *et al.*, 1979). The pedal ciliated cells of early embryos of *Helisoma* are innervated by a pair of serotonergic neurons, ENC1 (Diefenbach *et al.*, 1991, Diefenbach *et al.*, 1995). Application of exogenous 5-HT to whole embryos increased rotation rate, in addition to abolishing surge behavior (Diefenbach *et al.*, 1991). As the whole embryo studies suggested a role for 5-HT in regulating ciliary activity, a detailed examination of the direct actions of 5-HT was initiated using primary cell cultures of *Helisoma* ciliated cells.

Application of exogenous 5-HT to cultured ciliated cells increased CBF in a concentration-dependent fashion (Figure 6). An EC_{50} value was estimated to be between 0.1 μM and 1.0 μM 5-HT. At the highest concentration of 5-HT examined (100 μM), a 20-40% increase above basal CBF was characteristically observed (Figure 6). However, only 60% of ciliated cells examined responded to 100 μM 5-HT with an increase of at least 20%. In another 17% of the cells, a slight increase in CBF was observed (> 10%), whereas the remaining 23% of ciliated cells did not respond to 100 μM 5-HT.[3]

In the experiments described above, the effects of 5-HT were examined 10 min post-addition. In order to more fully characterize the cilio-excitatory effects of 5-HT, a detailed examination of the time course of 5-HT action was performed using a gravity-driven fast perfusion system. Application of exogenous 5-HT (10 μM) significantly increased CBF within 10 sec, followed by a maintained plateau (Figure 7). This effect was fully reversible as CBF values returned to basal levels within 30 sec of washout with HSD (Figure 7). Taken together, these results suggest that the cilio-excitatory effects of 5-HT are concentration-dependent, rapid, and fully reversible.

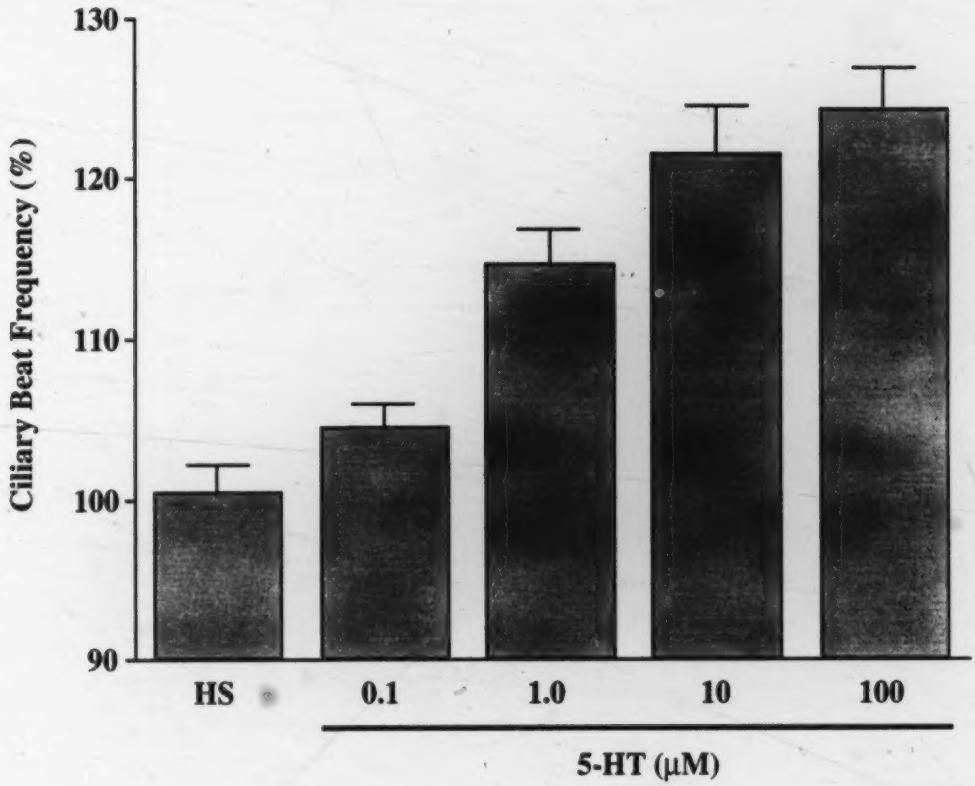


Figure 6: Concentration-dependent effects of 5-HT on CBF. 5-HT increased the frequency of ciliary beating in a concentration-dependent manner. Values are expressed as a percentage increase in CBF compared to basal levels. Each bar represents the average response of 28 to 47 cells, 10 min after addition of 5-HT or control vehicle (HS).

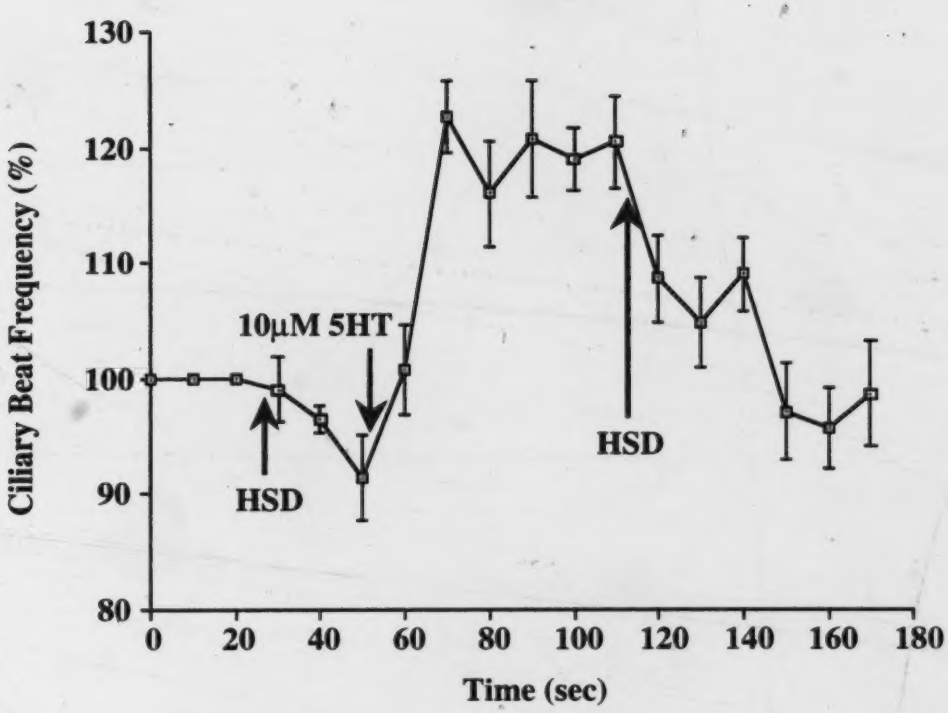


Figure 7: Time-course of 5-HT-stimulated CBF. Individual ciliated cells were perifused with HSD for 30 sec, 5-HT (10 μM) for 60 sec, and HSD for 60 sec. CBF values increased within 10 sec after addition of 5-HT, reached a plateau, and completely recovered to basal levels within 30 sec following washout with HSD. Values are normalized to the average of 3 CBF values recorded in HDM prior to the first perfusion with HSD. Arrows indicate the time of each treatment. Each data point represents the average response of 8 cells.

III. Role of Cyclic AMP [3]

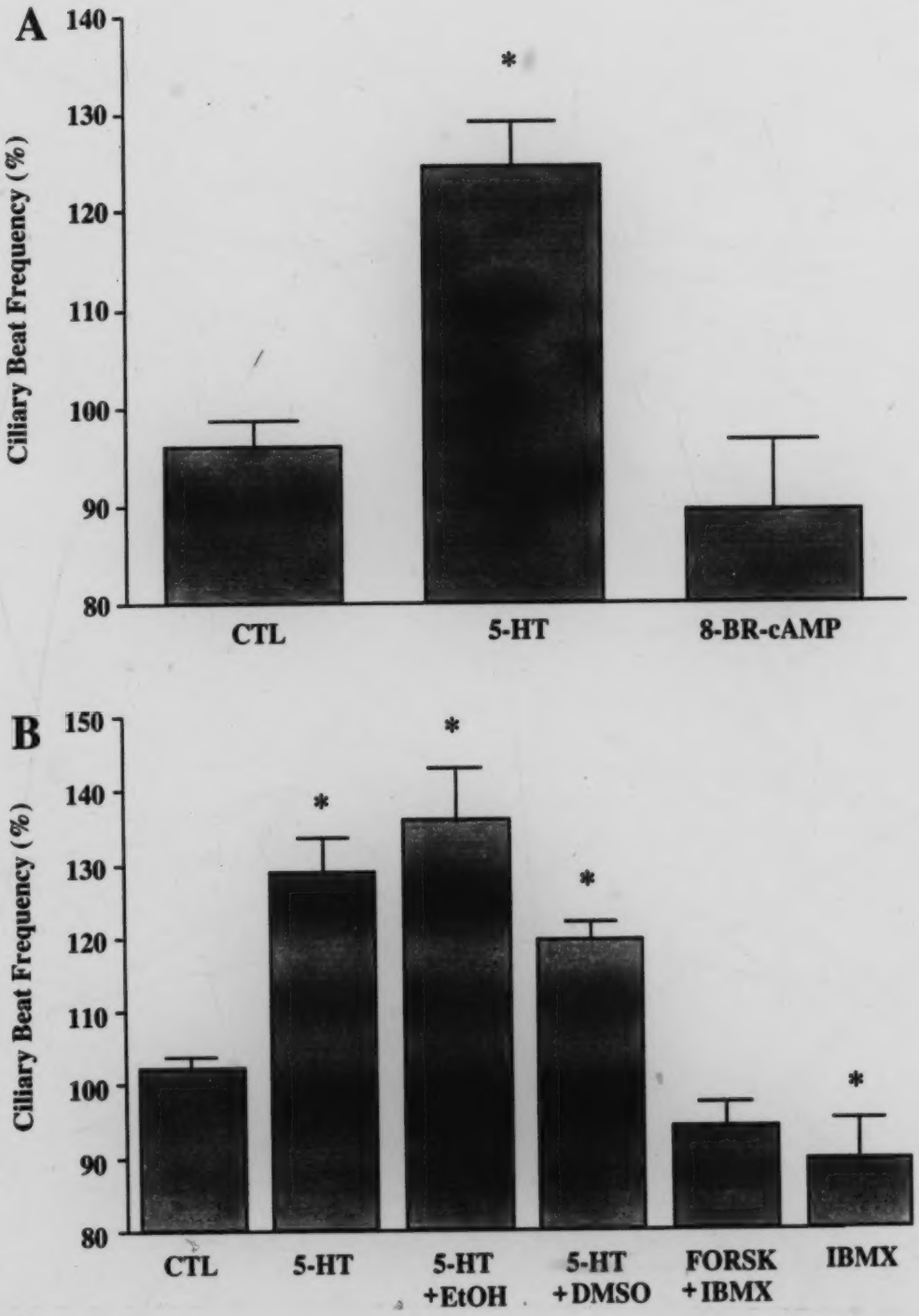
It has been suggested in another molluscan ciliary system, the extensively studied lateral gill cilia of *M. edulis* (Murakami 1983; 1987), that 5-HT-induced cilio-excitatory responses are mimicked by the elevation of cytoplasmic cyclic AMP. Furthermore, several neuronal responses to 5-HT in molluscs, including the 5-HT-induced depolarization in buccal neuron 19 of *Helisoma* (Price and Goldberg, 1993), involve the actions of cyclic AMP. I therefore tested the role of the cyclic AMP second messenger system in mediating 5-HT-induced cilio-excitation of *Helisoma*. The addition of 8-bromo-cyclic AMP (2 mM), a membrane permeable analog of cyclic AMP, did not mimic the increase in CBF induced by 100 μM 5-HT (Figure 8A).

Since membrane-permeable cyclic AMP analogs can vary in effectiveness, I further tested the cyclic AMP hypothesis in another manner. Co-application of forskolin (10 μM), an activator of adenylate cyclase, and IBMX (100 μM), an inhibitor of cellular phosphodiesterase, should effectively raise the intracellular cAMP concentration of cultured cells. Co-addition of these agents did not mimic the 5-HT-induced increase in CBF (Figure 8B). In fact, 100 μM IBMX induced a weak inhibition of basal CBF when presented alone ($p < 0.05$). To ensure that the lack of excitatory response to forskolin and IBMX was not related to the solvents, ethanol (0.05%) and DMSO (0.1%), control experiments were done to test the direct actions of these vehicle solutions. DMSO and ethanol had no significant effect on the basal CBF (not shown) nor on the 5-HT-stimulated CBF (Figure 8B). Taken together, these experiments suggest that the elevation of CBF induced by 5-HT is not mediated through a stimulation of the cyclic AMP second messenger system.

Since forskolin and IBMX, or IBMX alone, appeared to have weak inhibitory actions on basal CBF (Figure 8B), the cyclic AMP system may play an inhibitory role in the regulation of CBF. To test this hypothesis, 10 μM forskolin and 100 μM IBMX were

added in conjunction with 100 μM 5-HT (Figure 9). The stimulation of CBF induced by 5-HT was not affected by these compounds that elevate the intracellular cyclic AMP concentration. These experiments suggest that in embryonic *Helisoma* ciliated cells, cyclic AMP is not involved in the regulation of CBF.

Figure 8: Effects of treatments known to increase intracellular cyclic AMP concentrations on CBF. (A) Addition of the membrane-permeable analog 8-bromo-cyclic AMP (2 mM; 8-BR-CAMP)) resulted in a beat frequency that was not significantly different from control ($p>0.05$) whereas addition of 5-HT (100 μM) significantly increased CBF ($p<0.01$). Each bar represents the average response of 12 to 16 cells. (B) Co-addition of 10 μM forskolin (FORSK) and 100 μM IBMX did not increase CBF ($p>0.05$), whereas IBMX alone (100 μM) had a weak inhibitory effect ($p<0.05$). The addition of 5-HT alone or in combination with the vehicles ethanol (EtOH; 0.05%) and DMSO (0.1%) significantly increased CBF ($p<0.01$). Each bar represents the average response of 4 to 8 cells. Asterisks denote significant difference compared to control (CTL) value.


A
Ciliary Beat Frequency (%)
140
130
120
110
100
90
80
*
CTL
5-HT
8-BR-cAMP
B
Ciliary Beat Frequency (%)
150
140
130
120
110
100
90
80
*
*
*
*
CTL
5-HT
5-HT +EtOH
5-HT +DMSO
FORSK +IBMX
IBMX

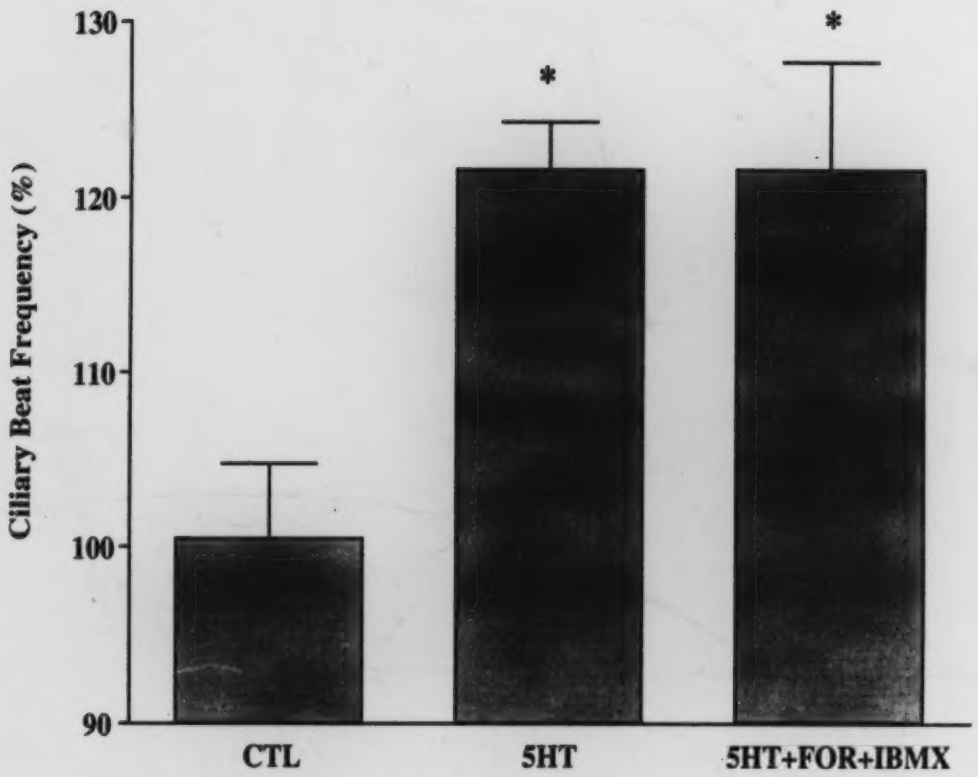


Figure 9: Increase in cyclic AMP does not affect the 5-HT-induced increase in CBF. Co-addition of 10 μM forskolin (FOR), 100 μM IBMX and 100 μM 5-HT resulted in an increase in CBF that was not significantly different from the increase observed due to addition of 5-HT alone ($p>0.05$). Each bar represents the mean response of 7 to 15 cells. Asterisks denote significant difference compared to control (CTL) value.

IV. Role of Ca^{2+} [3]

Similar to cyclic AMP, Ca^{2+} has been implicated as a cilio-excitatory intracellular messenger in many systems (see Introduction). Therefore, I tested the hypothesis that Ca^{2+} plays such a role in embryonic *Helisoma* ciliated cells by experimentally manipulating intracellular Ca^{2+} concentrations. Initially, cells were exposed to agents which would depolarize membrane potential and thereby activate voltage-gated Ca^{2+} channels. Lower concentrations of KCl (5-10 mM) induced a significant stimulation of CBF, whereas higher concentrations (20-25 mM) induced a significant inhibition of CBF (Figure 10A). Since addition of 10 mM NaCl had no effect on CBF (Figure 10B), the responses to KCl were likely a result of its depolarizing action, rather than the small shift in osmolarity that occurs with the addition of these compounds. The sodium channel agonist, veratridine, is another depolarizing agent that should indirectly increase the intracellular Ca^{2+} concentration. At 100 μM, veratridine mimicked the cilio-excitatory action of 10 mM KCl (Figure 10B). It is not known whether the increase in CBF induced by KCl or veratridine results directly from depolarization or indirectly through a rise in intracellular Ca^{2+}.

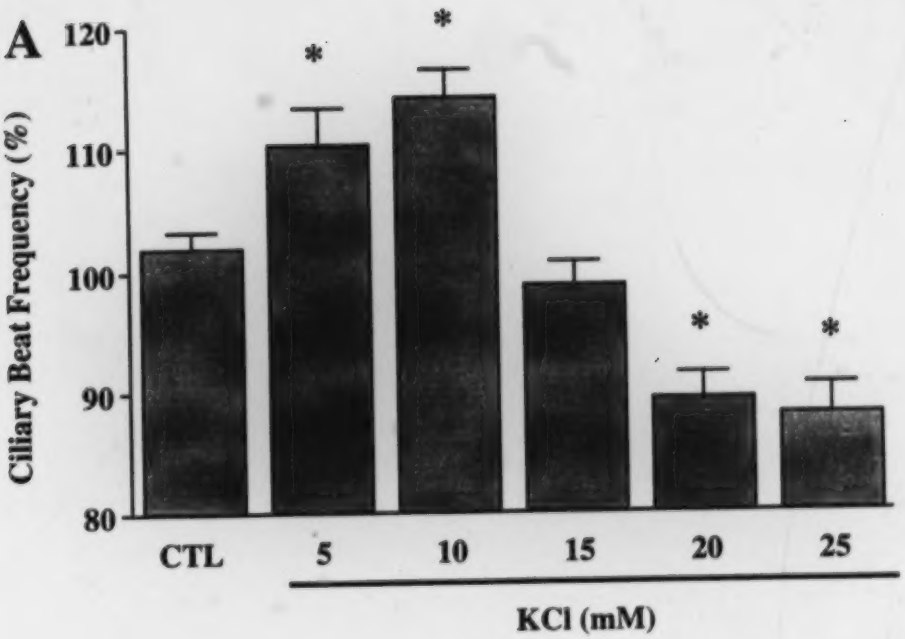
Pharmacological manipulations of intracellular Ca^{2+} were done to more directly test its involvement in the regulation of CBF. Addition of the Ca^{2+} ionophore, A23187 (10 μM), produced a significant increase in CBF that was similar to that induced by 5-HT (100 μM; Figure 11). Cilio-excitation was also observed in response to thapsigargin (0.1 μM; Figure 11), a compound that increases cytoplasmic Ca^{2+} by preventing the re-uptake of Ca^{2+} into intracellular storage compartments (Thastrup *et al.*, 1990). These results suggest that the 5-HT-induced increase in CBF may be mediated through an increase in intracellular Ca^{2+} concentration.

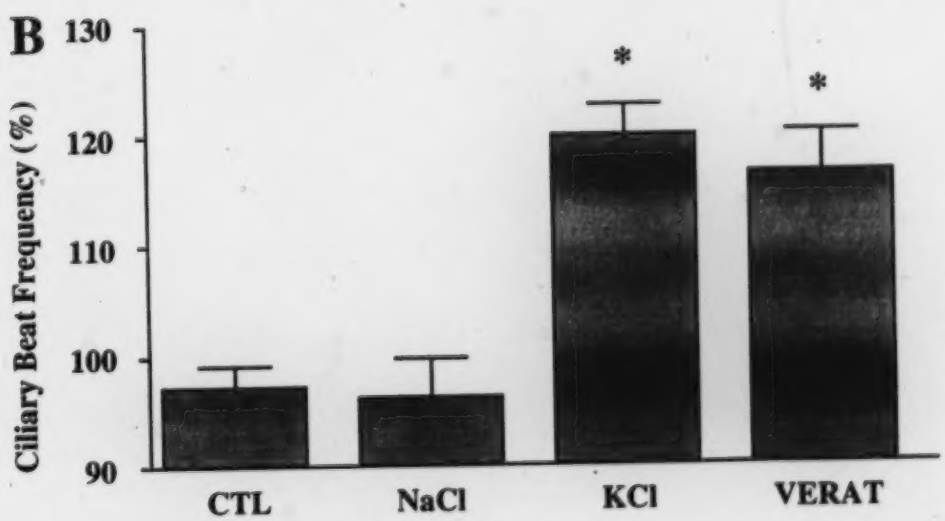
In order to more clearly establish a role for Ca^{2+} in 5-HT-induced cilio-excitation, the L-type Ca^{2+} channel agonist, BayK 8644, was tested for its ability to potentiate

increases in CBF in response to a sub-maximal concentration of 5-HT. BayK 8644 (100 nM) caused a significant increase in CBF ($p<0.05$; Figure 12), in a manner similar to that observed by application of 5-HT (1 μM). In addition, co-application of 5-HT and BayK 8644 further increased CBF (Figure 12). Taken together, these results suggest that Ca^{2+} influx through L-type Ca^{2+} channels may underlie 5-HT-induced cilio-excitation.

To obtain conclusive evidence for the involvement of Ca^{2+} influx through L-type Ca^{2+} channels, 5-HT was applied in combination with manipulations that prevent the influx of Ca^{2+}. In a normal Ca^{2+}-containing medium, 5-HT (100 μM) induced a robust increase in CBF (Figure 13A). When cells were incubated in a medium containing no Ca^{2+}, there was no change in the basal CBF. Furthermore, the 5-HT response was eliminated in the Ca^{2+}-free medium (Figure 13A). Similar results were obtained when blockers of L-type voltage-dependent Ca^{2+} channels were used to reduce Ca^{2+} influx (Figure 13B). Neither verapamil (1 μM) nor nifedipine (1 μM) caused a significant change in the basal CBF. However, both compounds significantly inhibited the effect of 5-HT on CBF (Figure 13B). Taken together, these results suggest that the 5-HT response involves the influx of extracellular Ca^{2+} through L-type channels. The subsequent elevation of intracellular Ca^{2+} may then act in the cytoplasm to stimulate ciliary beating.

Figure 10: Effects of depolarizing treatments on CBF. (A). The addition of KCl affected CBF in a biphasic concentration-dependent manner. Addition of 5 mM KCl ($p<0.05$) or 10 mM KCl ($p<0.001$) resulted in significant increases in CBF relative to control, whereas higher concentrations of KCl (20 or 25 mM; $p<0.001$) significantly reduced CBF. Each bar represents the mean response of 11 to 15 cells. (B). Addition of 10 mM KCl or 100 μM veratridine (VERAT) significantly increased CBF ($p<0.001$), whereas addition of 10 mM NaCl had no effect on CBF ($p>0.05$). Each bar represents the mean response of 6 to 22 cells. Asterisks denote significant difference compared to control (CTL) value.


A
120
110
100
90
80
Ciliary Beat Frequency (%)
*
*
*
*
CTL
5
10
15
20
25
KCl (mM)


B
130
120
110
100
90
Ciliary Beat Frequency (%)
*
*
CTL
NaCl
KCl
VERAT

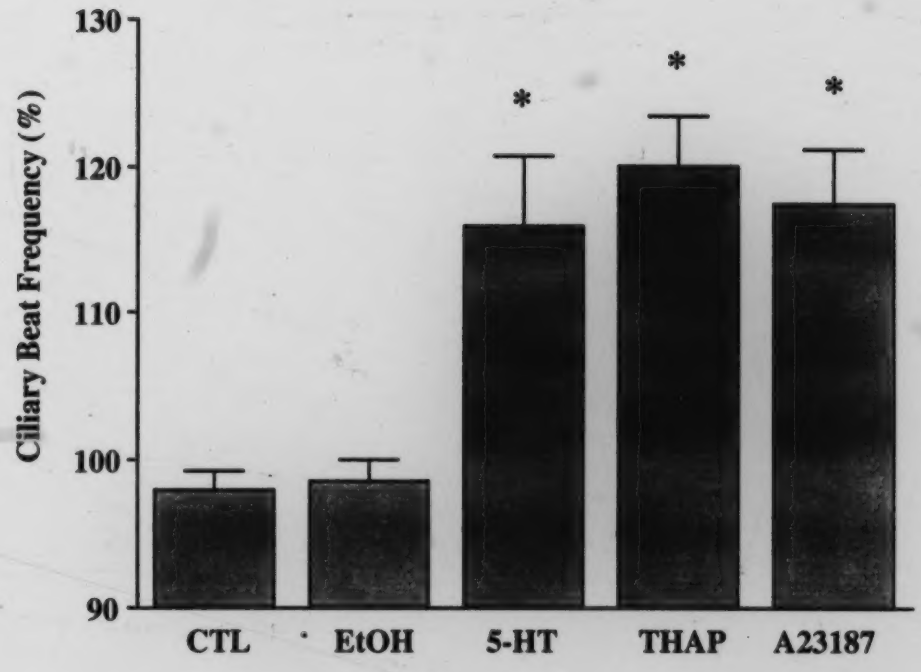


Figure 11: Effects of increasing intracellular calcium concentration on CBF. Addition of 0.1 μM thapsigargin (THAP; $p<0.001$) or 10 μM A23187 ($p<0.001$) mimicked the increase in CBF observed with addition of 100 μM 5-HT ($p<0.001$). In contrast, addition of the ethanol (EtOH; 0.05%) vehicle alone did not alter CBF as compared to control (CTL). Each bar represents the mean response of 13 to 23 cells. Asterisks denote significant difference compared to control value.

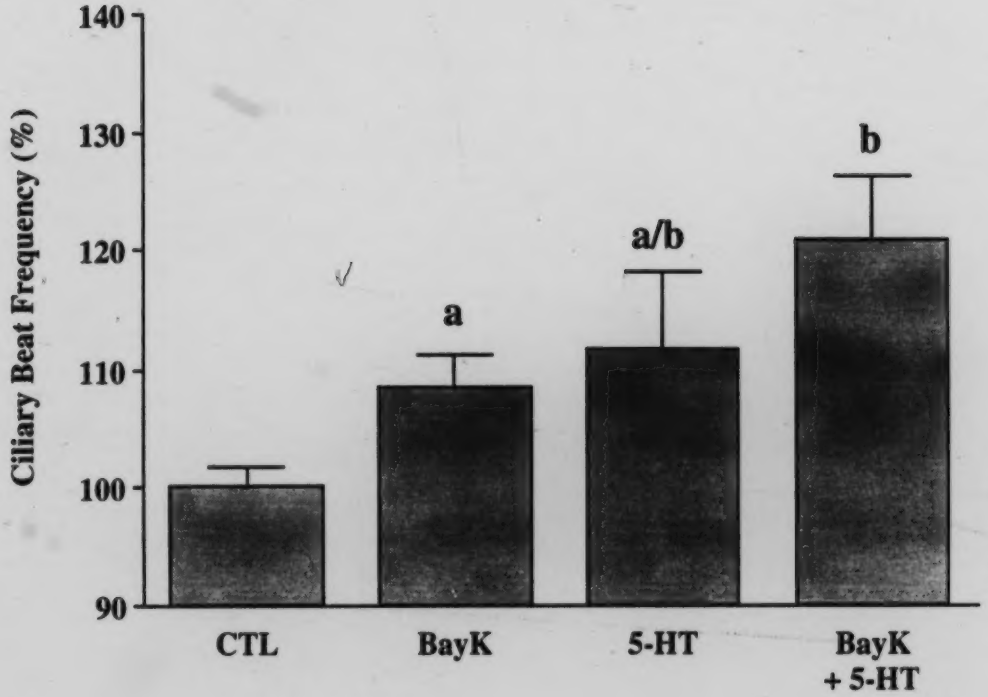
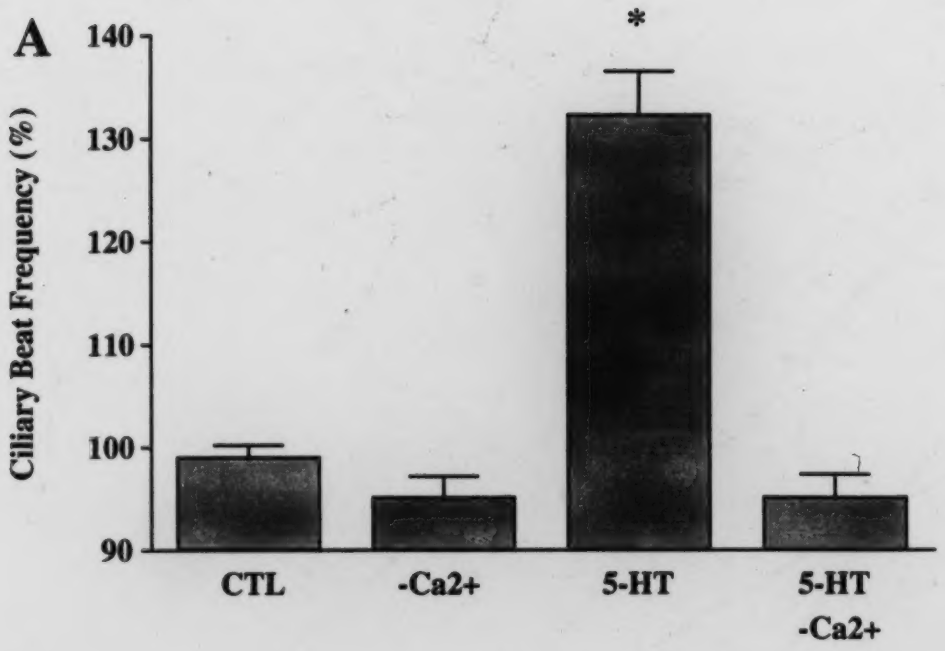
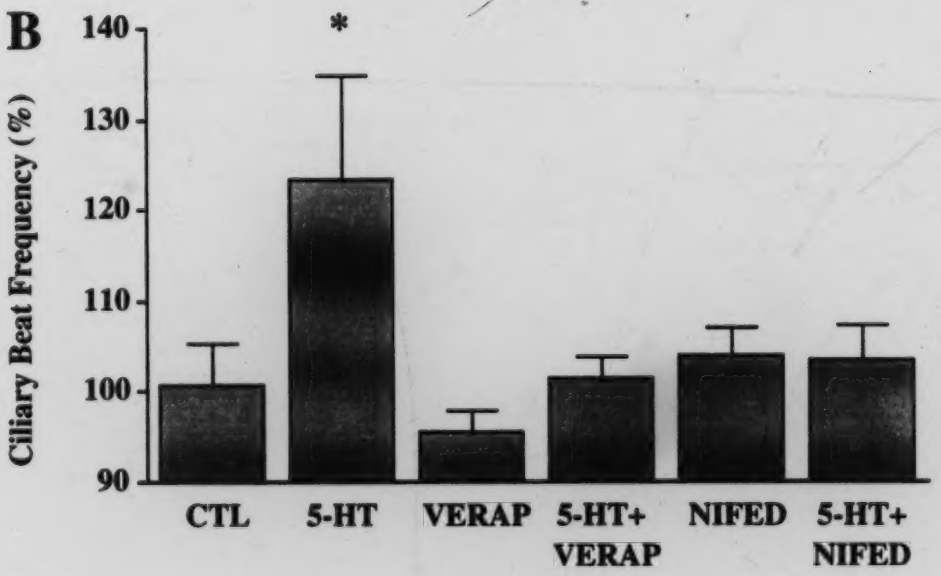


Figure 12: Effects of BayK 8644, an L-type Ca^{2+} channel agonist, on CBF. Addition of 5-HT (1 μM), BayK 8644 (10 nM), or co-addition of 5-HT and BayK 8644 significantly increase CBF compared to control (CTL) values. Each bar represents the average response of 6 to 28 cells. Statistically significant differences are indicated by different letter notations above each bar.

Figure 13: Effects of inhibiting calcium influx on CBF. (A) Ciliated cells bathed in a calcium-depleted medium (-Ca2+) beat at a normal frequency as compared to control cells (CTL). The addition of 100 μM 5-HT in a calcium-depleted medium caused no change in CBF, whereas 5-HT induced a significant increase in CBF ($p<0.001$) when cells were bathed in normal, calcium-containing medium. Each bar represents 14 to 18 cells. (B) Addition of 1 μM verapamil (VERAP) or 1 μM nifedipine (NIFED) alone did not alter CBF as compared to control (CTL). Co-incubation of 100 μM 5-HT with either verapamil or nifedipine abolished the increase in CBF that was observed when 5-HT was presented alone ($p<0.05$). All values are an average of 7 to 8 cells with the exception of the control value, which is an average of 3 cells. Asterisks denote significant difference compared to control value.


A
Ciliary Beat Frequency (%)
140
130
120
110
100
90
*
CTL
-Ca2+
5-HT
5-HT
-Ca2+


B
Ciliary Beat Frequency (%)
140
130
120
110
100
90
*
CTL
5-HT
VERAP
5-HT+
VERAP
NIFED
5-HT+
NIFED

V. Role of Protein Kinase C[4]

In many systems, 5-HT induces cellular responses through the activation of protein kinase C (PKC; Hill-Venning and Cottrell, 1992; Watts *et al.*, 1994; Taussig *et al.*, 1989). In addition, Ca^{2+} channel activity can be modulated through phosphorylation by PKC (DeRiemer *et al.*, 1985; Doerner and Alger, 1992). These findings prompted the hypothesis that 5-HT-induced cilio-excitation is mediated by a PKC-dependent pathway.

i) Effects of Diacylglycerol Analogs on CBF

Classical activation of PKC is via diacylglycerol (DAG) produced by metabolism of membrane phosphoinositols (Sanders-Bush *et al.*, 1990). In many systems, DAG analogs, such as OAG (Hill-Venning and Cottrell, 1992; Lapetina *et al.*, 1985) and DiC_8 (Kobayashi *et al.*, 1988; Lapetina *et al.*, 1985), are potent activators of PKC. Addition of the membrane permeable DAG analog, OAG, caused a transient increase in CBF (Figure 14). Within 1 min, CBF rose to 114% of pre-addition levels. However, this effect was absent by 10 min post-addition of OAG (Figure 14). In contrast, 5-HT induced a sustained increase in CBF to levels ranging between 120-150% of basal values (Figure 14; Christopher *et al.*, 1996; Goldberg *et al.*, 1994). DMSO (0.1%), the vehicle for OAG, had no effect on CBF.

DiC_8, another DAG analog, also mimicked the 5-HT-stimulated increase in CBF in a similar manner to OAG (Figure 15). Again, the effect of DiC_8 was transient; the increase in CBF persisted for 5 min post-addition, but was absent by 10 min post-addition (Figure 15). These results suggest that a DAG-mediated signaling pathway may underlie 5-HT-stimulated increases in CBF.

Interactions between the DAG analog, DiC_8, and 5-HT were examined to determine whether PKC activation is a component of, rather than separate from, 5-HT-induced cilio-excitation. Ciliated cells incubated in the presence of DiC_8 for 2 min

resulted in an increase in CBF. Moreover, a further increase in CBF was observed when examined 2 min post-addition of 5-HT (Figure 16A). In contrast, initial addition of 5-HT increased CBF in a characteristic manner, whereas subsequent addition of DiC_8 did not increase CBF to a level over and above the 5-HT-induced increase in CBF (Figure 16B). The absence of additivity is consistent with the hypothesis that PKC is involved in mediating the 5-HT response.

ii) Effects of Protein Kinase C Inhibitors on 5-HT-Stimulated CBF

PKC inhibitors were utilized to further examine PKC involvement in 5-HT-induced increases in CBF. Bisindolylmalemide inhibits PKC activity in guinea pig tracheal muscle (Watts *et al.*, 1994) and rat cerebellar granule cells (Pizzi *et al.*, 1996). Bisindolylmalemide acts through competition with the ATP binding site within the catalytic domain of PKC (Toullec *et al.*, 1991). Pre-incubation of ciliated cells with bisindolylmalemide (10 nM or 100 nM) or the vehicle control, DMSO (0.1%), for 10 min did not alter ciliary beating (Figure 17). Subsequent to control vehicle application, 5-HT induced a characteristic increase in CBF when measured 10 min post-addition. However, bisindolylmalemide significantly inhibited the 5-HT-stimulated increase in CBF in a concentration-dependent manner (Figure 17; $p<0.05$). These results suggest that a PKC pathway mediates the cilio-excitatory effect of 5-HT.

The specificity of bisindolylmalemide inhibition was examined by pre-incubation of ciliated cells with bisindolylmalemide for 10 min, followed by addition of DiC_8. Bisindolylmalemide (100 nM) significantly inhibited the DiC_8-induced increases in CBF (Figure 18), suggesting that bisindolylmalemide acted specifically on a DAG/PKC pathway at the concentrations tested.

Another specific PKC inhibitor, Calphostin C, was used to confirm a role for PKC in mediating 5-HT's effects. Calphostin C inhibits PKC activity through competition with

the DAG/phorbol ester binding site within the regulatory domain (Kobayashi *et al.*, 1989). Ciliated cells preincubated for 10 min with Calphostin C (10 nM) or DMSO (0.1%) did not demonstrate an alteration in CBF. However, following exposure to Calphostin C, addition of 5-HT did not cause the significant increase in CBF that was observed under control conditions (Figure 19). These results, together with the results from experiments using bisindolylmalemide, suggest that a PKC pathway is involved in 5-HT-stimulated cilio-excitation.

iii) Role of Phorbol Esters

As a role for PKC was implicated by the above findings, the effect of a phorbol ester on CBF was tested. Phorbol esters are used in numerous systems to activate PKC (DeRiemer *et al.*, 1985; Hill-Venning and Cottrell, 1992; Geiges *et al.*, 1997). Whereas a characteristic increase in CBF was observed upon addition of 5-HT (Figure 20B), TPA did not stimulate CBF at all 3 concentrations tested (0.1, 10, 1000 nM; Figure 20A). These findings were contrary to the effect observed upon addition of either DAG analog. As the cilio-excitatory effects of both DiC_8 and OAG were transient, the actions of TPA at earlier time points were examined. At 1, 2, 3, 5, and 10 min post-addition, application of TPA (1 μM) or DMSO (0.1%) did not alter CBF. In contrast, cilio-excitation upon 5-HT addition was observed at all time points examined (Figure 20C).

Different isoforms of PKC can have different sensitivities to phorbol ester activation (Geiges *et al.*, 1997; Gibson and Logan, 1992). Therefore, phorbol dibenzoate (PDBn), another active phorbol ester, was used to verify the lack of cilio-excitation by TPA. Similar to the effects of TPA, PDBn (10 μM) did not cause an increase in CBF (Figure 21). Taken together, the effect of TPA and PDBn on CBF suggest that application of phorbol esters are unable to mimic 5-HT-stimulated ciliary activity.

In both bovine and rabbit tracheal ciliary systems, activation of PKC results in a significant decrease in CBF (Salathe *et al.*, 1993; Kobayashi *et al.*, 1988). While TPA and PDBn did not alter CBF significantly from basal levels nor mimic the 5-HT-induced cilio-excitation, phorbol esters may have inhibitory actions on 5-HT-stimulated ciliary activity. Thus, an examination of the effect of TPA on 5-HT-stimulated CBF was performed. Ciliated cells were incubated with TPA (1 μM) or DMSO (0.1%) for 10 min, followed by addition of 5-HT (100 μM). In both cases, a robust increase in CBF was observed (Figure 22A), suggesting that pre-incubation with TPA does not alter 5-HT effects. In addition, co-addition of TPA and 5-HT similarly resulted in an increase in CBF (Figure 22B). These results strongly suggest that phorbol esters neither mimic nor inhibit 5-HT-stimulated increases in CBF.

A strong dependence on phosphatidylserine is a common feature of several members of the PKC family (Nishizuka, 1992). The synthetic form of phosphatidylserine, SC-9 (*N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide), is an agent capable of activating Ca^{2+}-dependent isoforms of PKC (Nishino *et al.*, 1986; Bargon *et al.*, 1992). CBF was unchanged in the presence of SC-9 (10 μM) or DMSO (0.1%; vehicle control) for up to 10 min following addition (Figure 23). However, a robust 5-HT-stimulated increase in CBF was observed at all time points examined (100 μM; Figure 23). These results suggest that the isoform of PKC underlying 5-HT-induced increases in CBF may not require phosphatidylserine for activation.

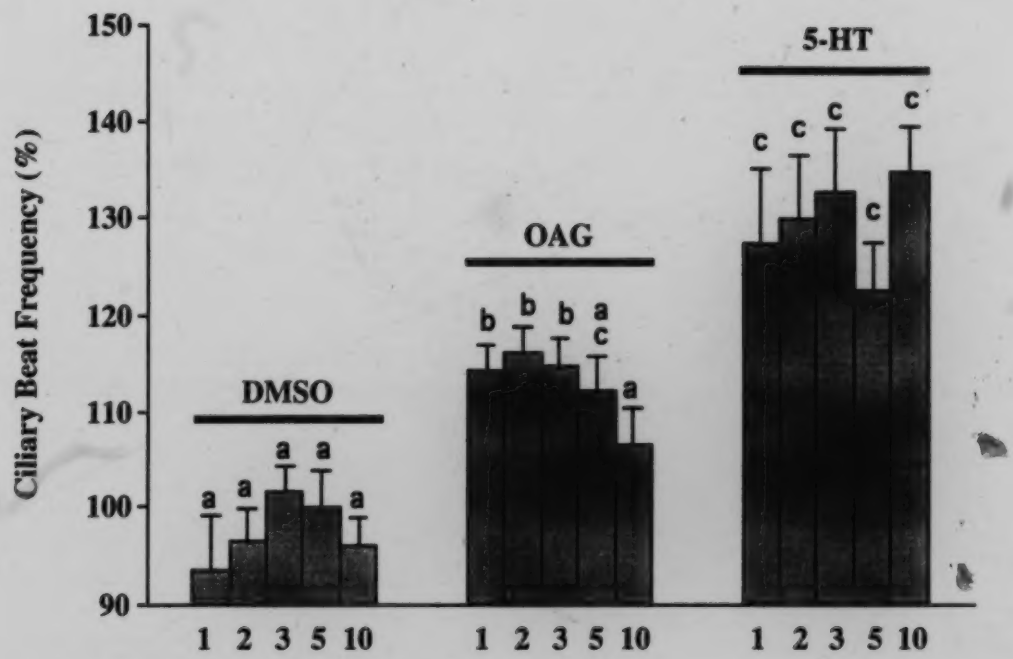


Figure 14: Effect of OAG, a membrane-permeable diacylglycerol analog, on CBF. OAG (100 μM) significantly increased CBF within 1 min of addition ($p < 0.05$). By 10 min post-addition, CBF levels had returned to baseline values. Addition of the vehicle control, DMSO (0.1%) did not affect CBF, whereas addition of 5-HT (100 μM) produced a sustained increase in CBF that developed in less than 1 min. Each bar represents the mean response of 6 to 15 cells. Within each treatment, CBF values are statistically similar. Statistically significant differences are indicated by different letter notations above each bar.

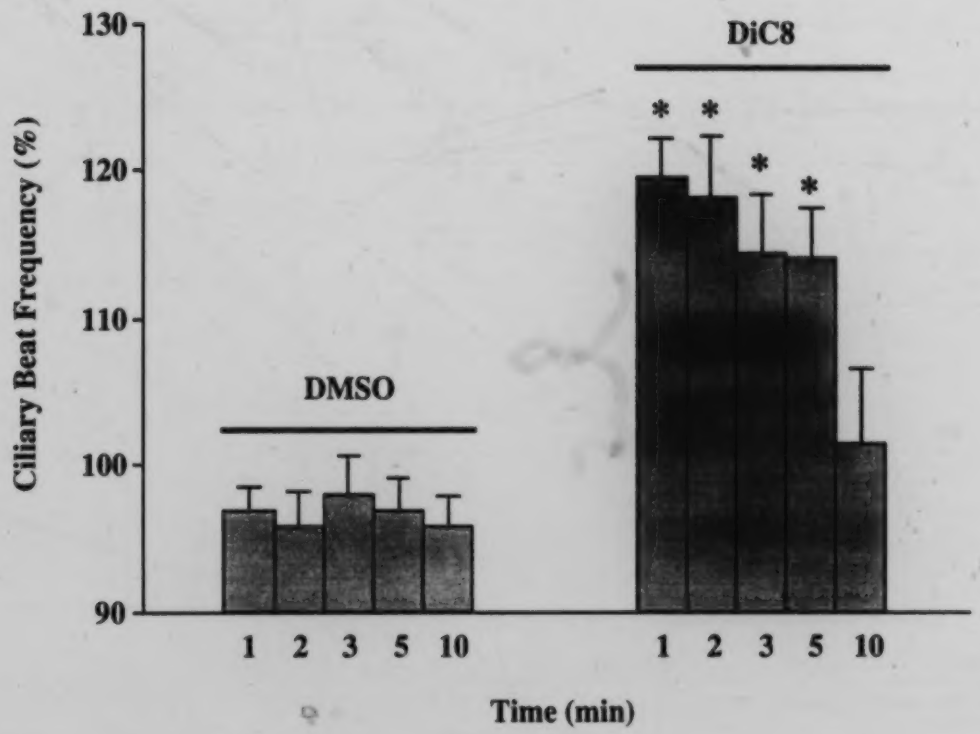
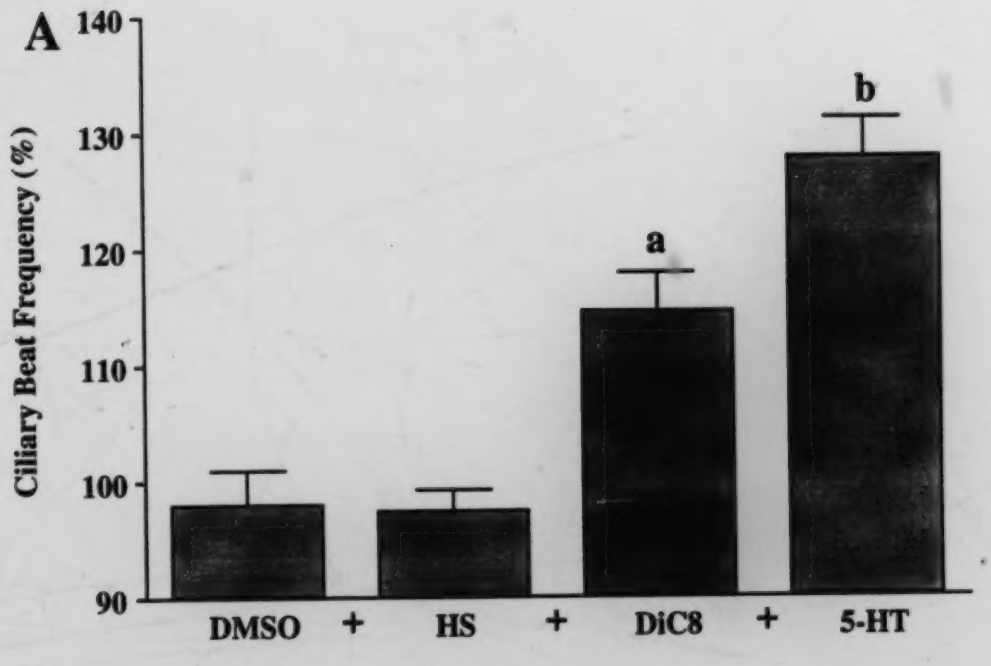
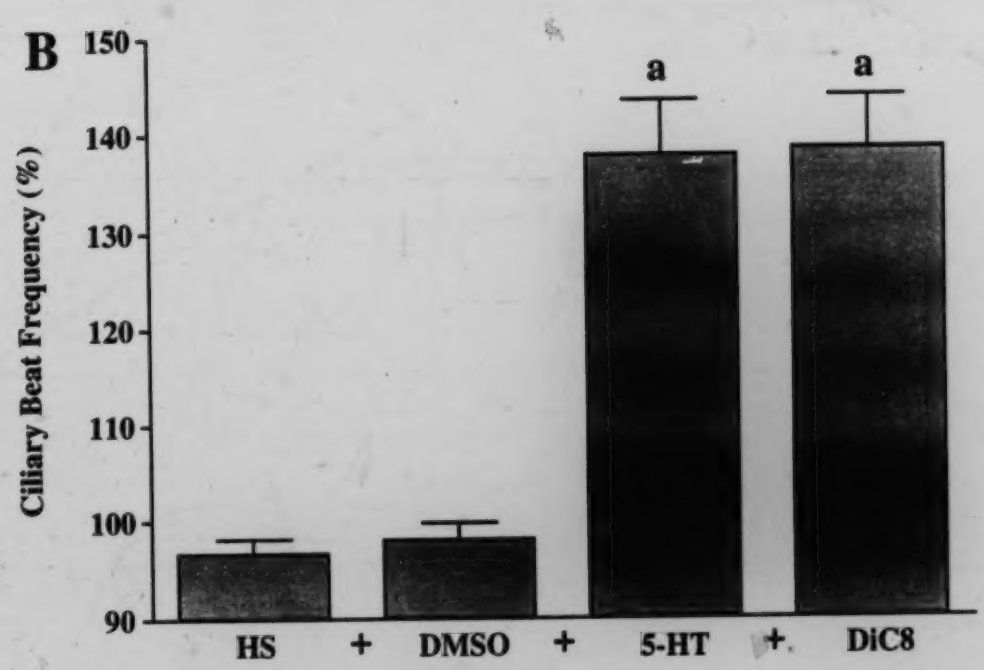


Figure 15: Effect of DiC_8, a diacylglycerol analog, on CBF. DiC_8 (100 µM) induced a transient increase in CBF, whereas DMSO (0.1%) did not affect CBF at all time points examined. Each bar represents the mean response of 9 to 20 cells. Asterisks denote statistically significant differences compared to the matching time point between treatment groups ($p<0.05$).

Figure 16: Lack of additivity between DiC_8- and 5-HT- stimulated CBF. Ciliated cells were exposed to a test agent for 2 min, followed by determination of CBF and addition of the next drug. Total time of each experiment was 8 min. (A) Initial exposure to DiC_8 (100 μM) caused a significant ($p<0.05$) increase in CBF. Subsequent addition of 5-HT (100 μM) further increased CBF. Each bar represents the mean response of 13 cells. (B) Initial exposure to 5-HT (100 μM) significantly increased CBF ($p<0.05$), whereas subsequent addition of DiC_8 did not produce a further increase in CBF. Each bar represents the mean response of 11 cells. Statistically significant differences are indicated by different letter notations above each bar.


A
Ciliary Beat Frequency (%)
140
130
120
110
100
90
a
b
DMSO + HS + DiC8 + 5-HT


B
Ciliary Beat Frequency (%)
150
140
130
120
110
100
90
a
a
HS + DMSO + 5-HT + DiC8

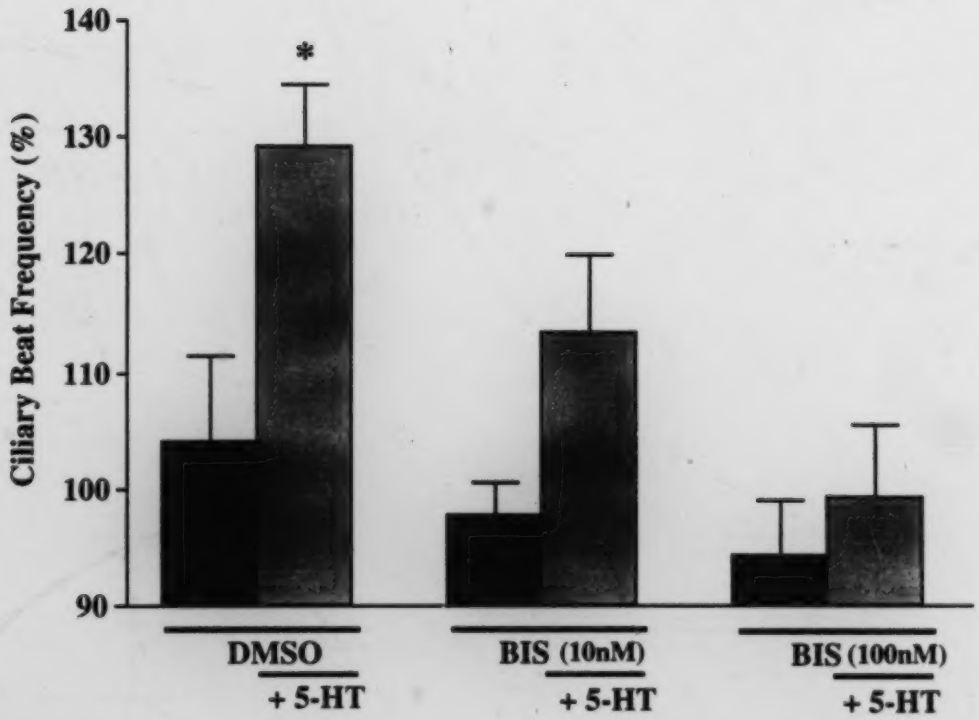


Figure 17: Effect of bisindolylmalemide, a specific PKC inhibitor, on 5-HT-stimulated CBF. DMSO (0.01%, vehicle control) or bisindolylmalemide (10 nM and 100 nM), added 10 min prior to determination of CBF, did not affect basal CBF. A characteristic increase in CBF in response to 5-HT (100 μM) was observed in cells pretreated with DMSO. In contrast, prior exposure to bisindolylmalemide significantly reduced 5-HT-induced ciliary activity. Each bar represents the mean response of 9 to 11 cells, except DMSO, where n=3. Asterisk denotes statistically significant differences compared to vehicle control and matched time points between treatments ($p<0.05$).

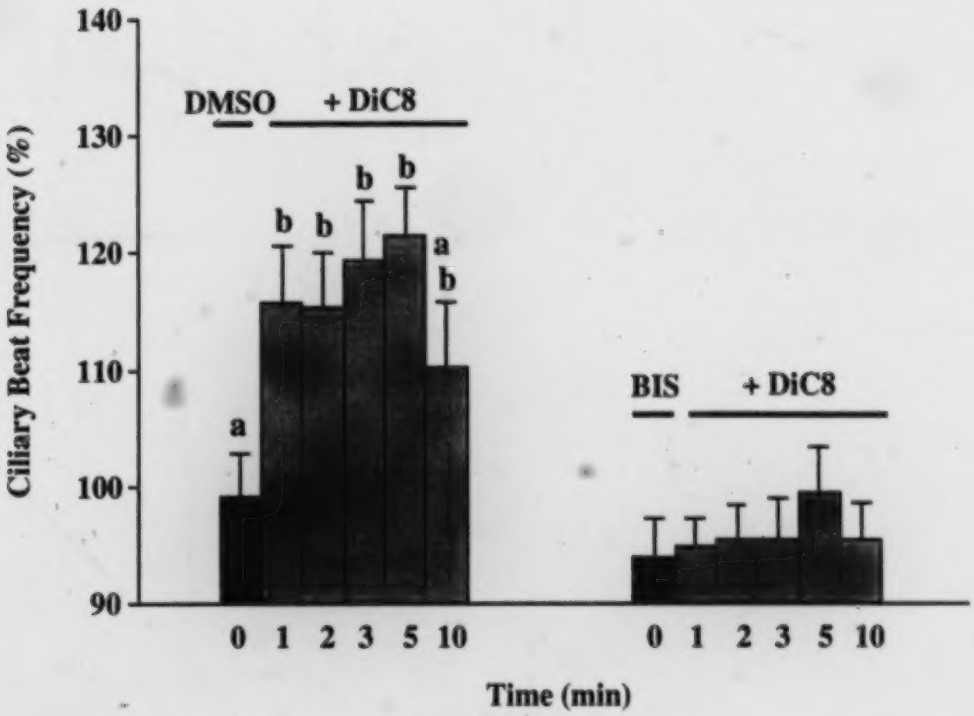


Figure 18: Effect of bisindolylmalemide on the cilio-excitatory action of DiC_8. Exposure of ciliated cells to DMSO (0.01%) or bisindolylmalemide (100 nM) for 10 min did not affect basal CBF. DiC_8 added following DMSO exposure significantly increased CBF ($p<0.05$) for up to 5 min. The cilio-excitatory responses of DiC_8 were significantly inhibited by pre-exposure to bisindolylmalemide at all time points examined. Each bar represents the mean response of 5 to 10 cells. Statistically significant differences are indicated by different letter notations above the bar. Matched time points between treatment groups following exposure to DiC_8 were significantly different ($p<0.05$).

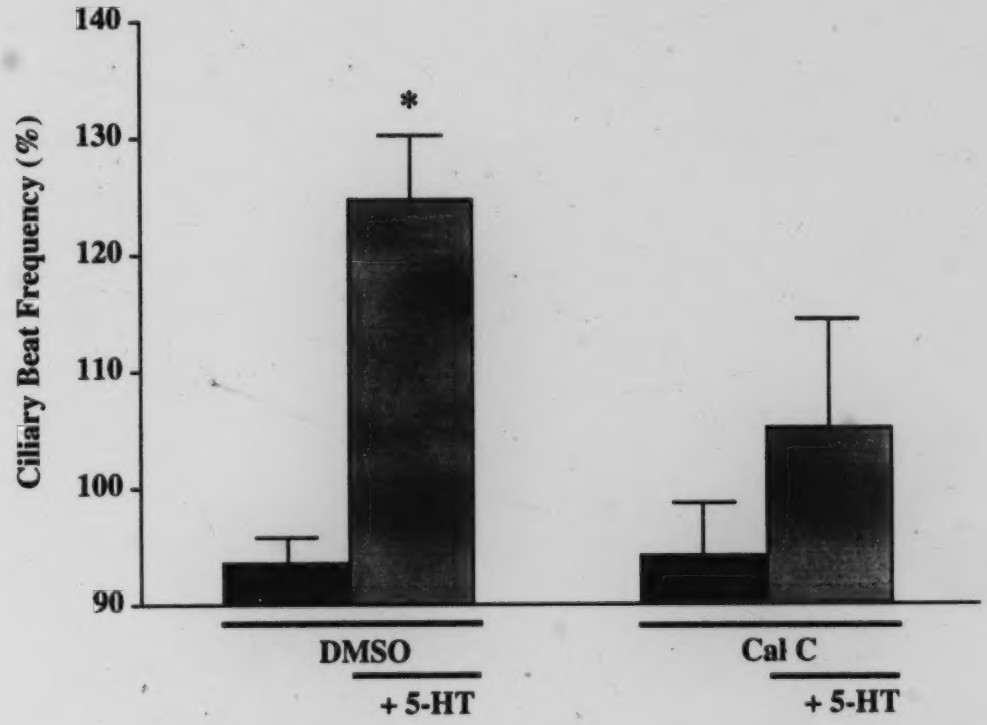
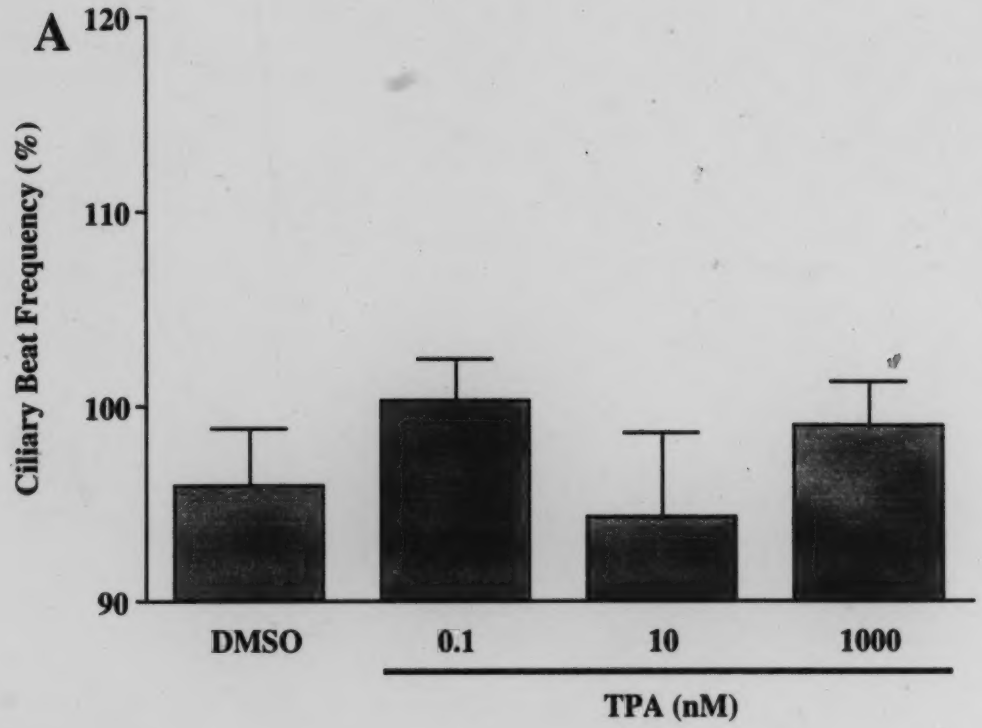
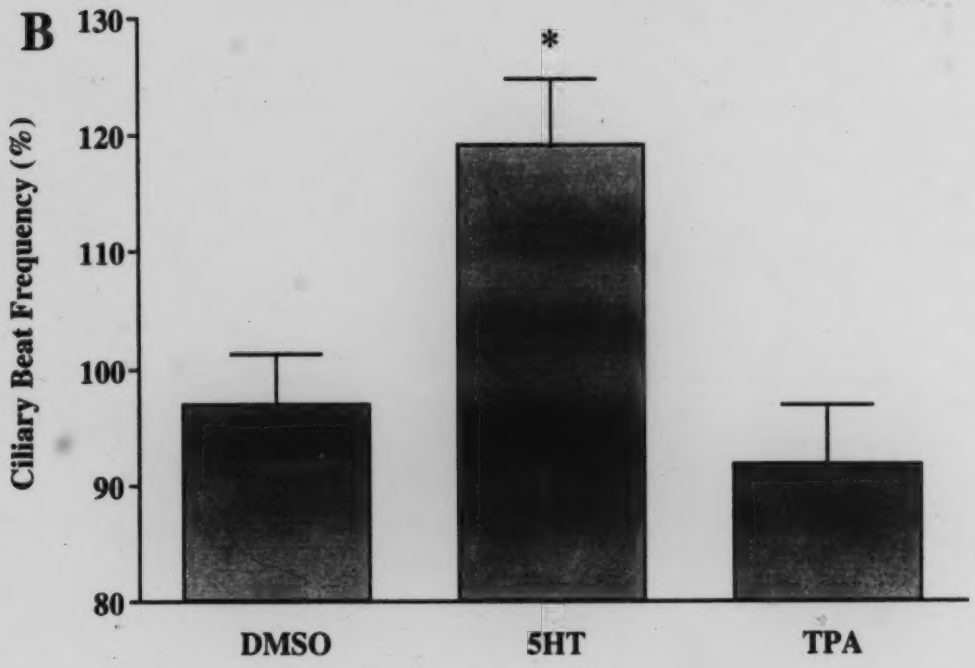
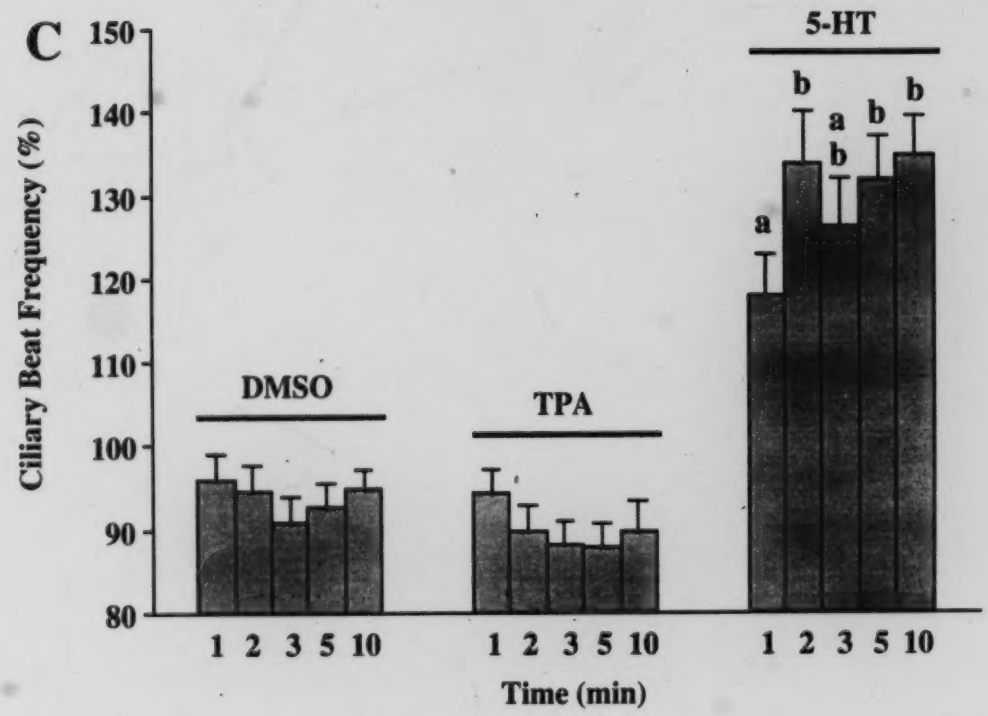


Figure 19: Effect of Calphostin C on 5-HT-induced cilio-excitation. Ciliated cells were exposed to DMSO (0.1%, vehicle control) or Calphostin C (Cal C; 10 nM) for 10 min in the presence of direct light, followed by 5-HT challenge (100 μM). Addition of DMSO or Calphostin C did not affect basal CBF. Cilio-excitation was observed upon 5-HT addition following DMSO pretreatment, but was prevented by pretreatment with Cal C. Each bar represents the mean response of 8 to 13 cells. Asterisk denotes significance compared to the vehicle control and matched time points between separate treatments ($p<0.05$).

Figure 20: Effect of TPA, an activator of PKC, on CBF. Ciliated cells were exposed to each pharmacological agent for 10 min, followed by determination of CBF. (A) At the 3 concentrations tested (0.1, 10, 1000 nM), TPA (phorbol 12-myristate 13-acetate) had no significant effect on CBF, as compared to the vehicle control treatment (DMSO; 0.1%). Each bar represents the mean response of 8 to 10 cells.(B) In a different set of experiments, a robust increase in CBF was observed upon addition of 5-HT (100 μM), whereas addition of TPA (1 μM) did not increase CBF. Each bar represents the mean response of 7 to 12 cells. Asterisk denotes statistical significance compared to vehicle control. (C) Each pharmacological agent was added, followed by determination of CBF at 1, 2, 3, 5, and 10 min post- addition. At all time points, DMSO (vehicle control; 0.1%) and TPA (1 μM) did not increase CBF. In contrast, a characteristic increase in CBF was observed upon 5-HT (100 μM) exposure at all time points examined. Each bar represents the mean response of 6 to 13 cells. Statistically significant differences between matched time points between treatment groups and within the 5-HT treatment are indicated by different letter notations above the bar.


A
120
110
100
90
Ciliary Beat Frequency (%)
DMSO
0.1
10
1000
TPA (nM)


B
130
120
110
100
90
80
*
Ciliary Beat Frequency (%)
DMSO
5HT
TPA


C
Ciliary Beat Frequency (%)
150
140
130
120
110
100
90
80
DMSO
TPA
5-HT
a
b
a
b
b
b
1 2 3 5 10
1 2 3 5 10
1 2 3 5 10
Time (min)

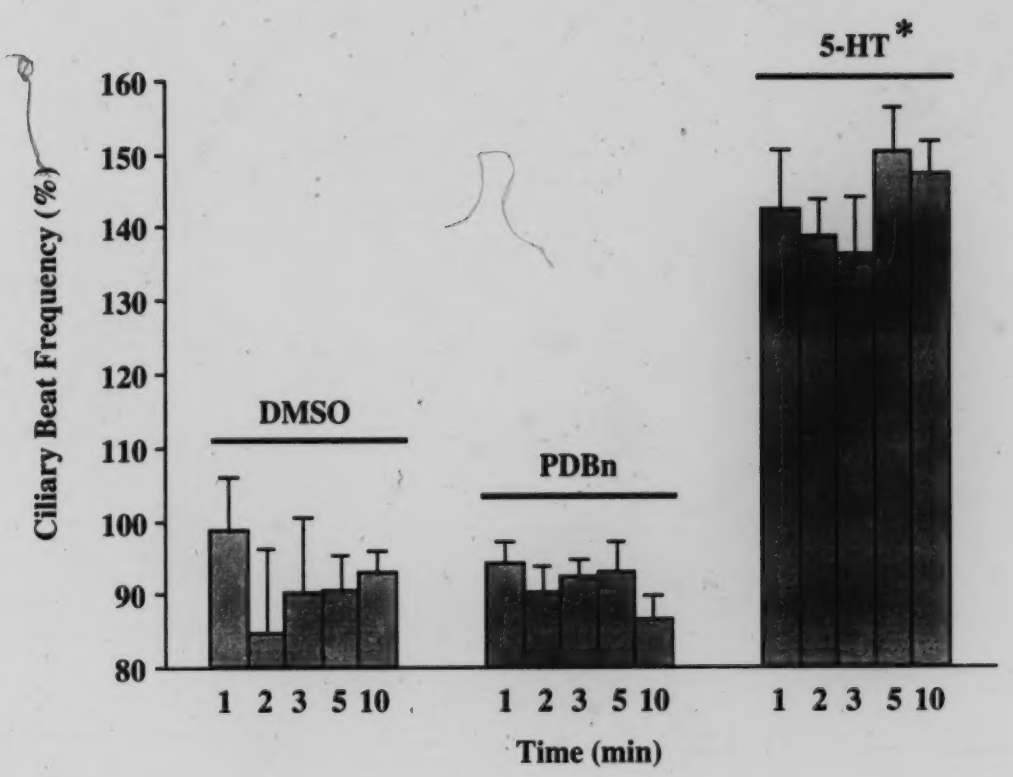
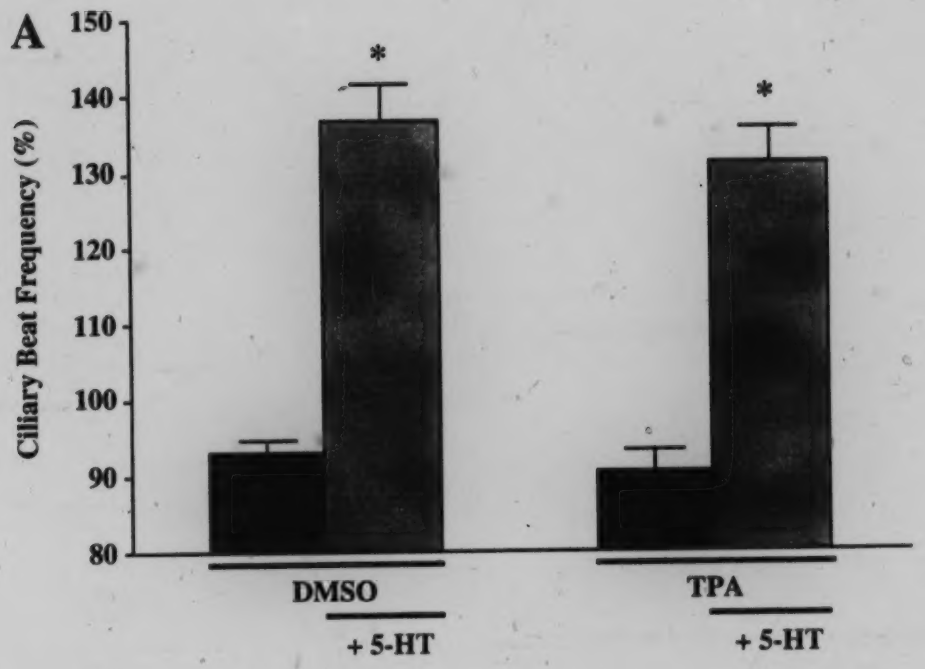
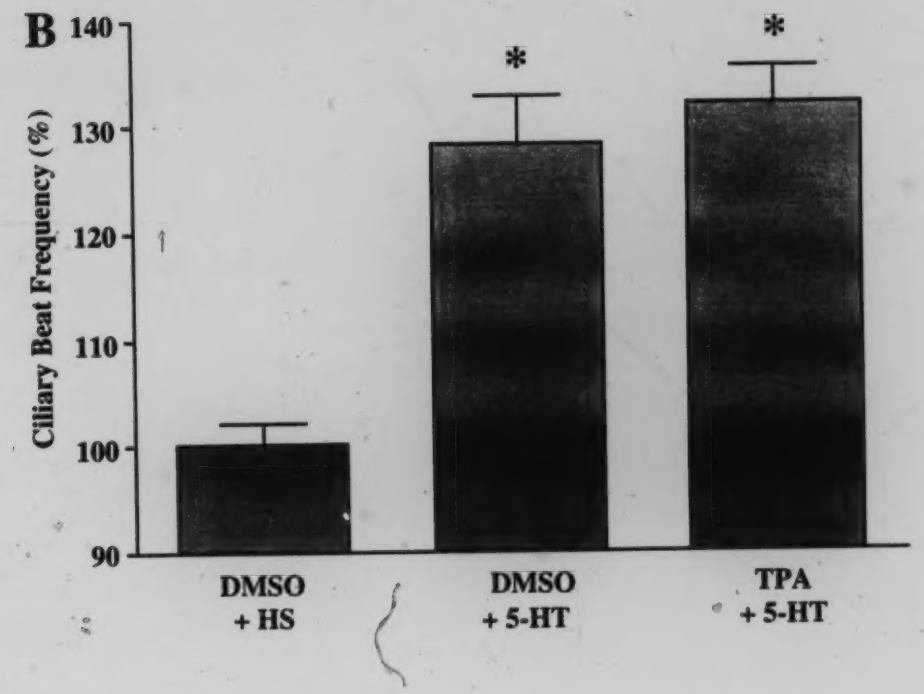


Figure 21: Effect of phorbol dibenzoate, an activator of PKC, on CBF. CBF was determined 1, 2, 3, 5, and 10 min post-addition of DMSO (vehicle control; 0.1%), phorbol 12β, 13α-dibenzoate (PDBn; 10 μM) or 5-HT (100 μM). Each bar represents the mean response of 4 to 8 cells. Asterisk denotes significant difference between all matched time points in the separate treatment groups ($p<0.05$). There was no difference between each time point within treatment groups.

Figure 22: Effect of TPA on 5-HT-stimulated CBF. (A) Ciliated cells were exposed to TPA (1 μM) or DMSO (0.1%) for 10 min, followed by addition of 5-HT (100 μM) for 10 min. Addition of TPA or DMSO did not affect basal or 5-HT-stimulated CBF. Each bar represents the mean response of 13 to 15 cells. Asterisks denote significant difference compared to pre-5-HT values. (B) Co-addition of TPA (1 μM) or DMSO (0.1%) with 5-HT (100 μM) resulted in a robust increase in CBF in both cases. CBF values were determined 10 min after addition of each treatment. Each bar represents the mean response of 6 to 11 cells. Asterisks represent statistical significance compared to control (DMSO + HS).


A
Ciliary Beat Frequency (%)
150
140
130
120
110
100
90
80
*
*
DMSO
+ 5-HT
TPA
+ 5-HT


B
Ciliary Beat Frequency (%)
140
130
120
110
100
90
*
*
DMSO
+ HS
DMSO
+ 5-HT
TPA
+ 5-HT

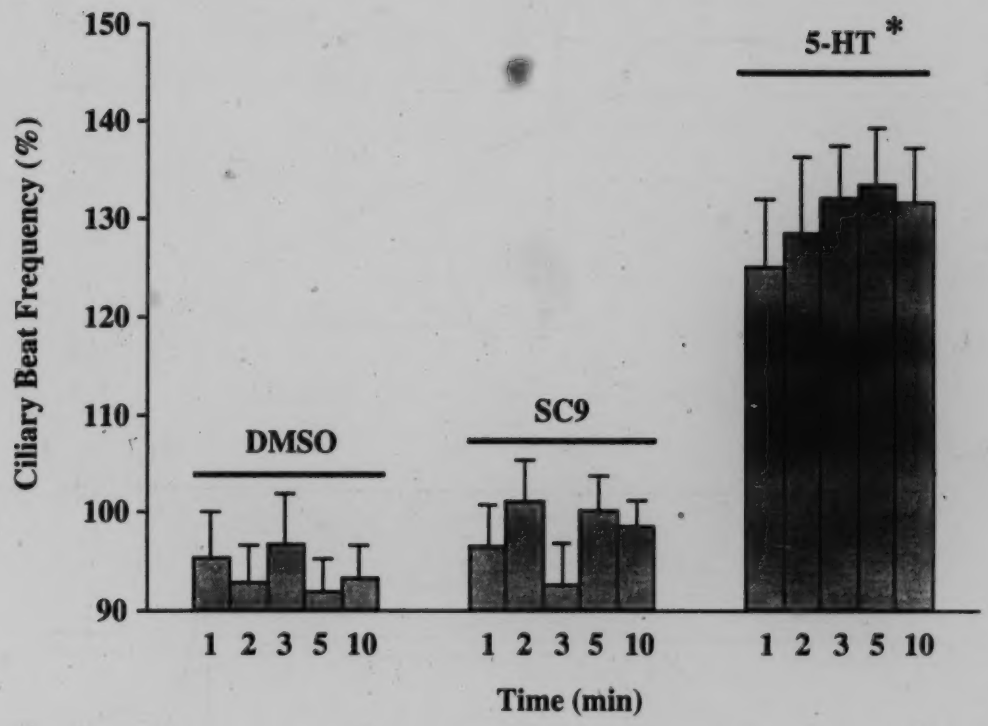


Figure 23: Effect of SC-9, an activator of Ca^{2+}/phosphatidylserine-dependent PKC, on CBF. CBF was determined 1, 2, 3, 5, and 10 min post-addition of DMSO (vehicle control; 0.1%), SC-9 (10 μM), or 5-HT (100 μM). SC-9 did not mimic the 5-HT-induced cilio-excitation. Each bar represents the mean response of 8 to 12 cells. Asterisk denotes significant difference between matched time points between the treatment groups ($p<0.05$). There was no difference between time points within individual treatment groups.

VI. Quantification of Intracellular Ca^{2+} Concentrations in Ciliated Cells

The experiments described in section IV suggest that an increase in intracellular Ca^{2+} concentration mediates 5-HT-stimulated cilio-excitation. To further characterize the role of Ca^{2+}, quantification of spatial and temporal changes of free intracellular Ca^{2+} concentrations in response to 5-HT is necessary. As a first step towards this goal, basal levels of intracellular Ca^{2+} were determined using the Ca^{2+}-sensitive fluorescent indicator, Fura-2. As the cultured *Helisoma* ciliated cells presented a unique set of problems for Fura-2 calcium imaging experiments, significant effort was directed towards establishing the appropriate conditions to achieve reliable measurements. The experiments involved in establishing these conditions, in addition to providing important information on basal Ca^{2+} concentrations, are described below. They serve as the foundation for future experiments on the spatio-temporal measurement of 5-HT-induced changes in intracellular Ca^{2+} concentrations.

i) Loading Ciliated Cells with Fura-2

Two forms of the Fura-2 dye exist, a membrane-permeable ester and a membrane-impermeable salt that must be injected into cells (Moore *et al.*, 1990). As the ciliated cells are extremely sensitive to micropipette penetration (Collins, Young, and Goldberg, unpublished results), the membrane-permeable ester form was chosen for this study. The ester form passes through the cell membrane and is subsequently cleaved by endogenous esterases, resulting in an impermeant, active, Ca^{2+}-binding dye (Kao, 1994). Experiments were performed to determine optimal loading conditions, including Fura-2 concentration, incubation time, and washout time. In initial attempts, ciliated cells were incubated with 4 μM Fura-2-AM (dissolved in DMSO, final concentration 0.1% in HDM) for 60 min. This incubation was followed by 3 x 15 min washes in HS. Since HDM contains the pH indicator phenol red, which has inherent fluorescent properties (Moore *et al.*, 1990), HS

was used for both washes and actual imaging experiments. With this protocol, ciliated cells with actively beating cilia were not observed. In most cases, cells exhibiting the characteristic shape and granular cytoplasmic appearance of ciliated cells were found, yet cilia were not readily apparent. Rarely, ciliated cells with arrested cilia were observed. It was hypothesized that a long-term (60 min) incubation in 0.1% DMSO, the solvent vehicle for Fura-2, was detrimental to ciliary activity. Using time-lapse videomicroscopy, CBF was therefore measured during a 60 min incubation in 0.1% DMSO. This concentration of DMSO did not affect CBF over the entire 60 min period (Figure 24). In contrast, CBF declined significantly when cells were exposed to HS (Figure 24; 25A). This effect was not due to the mechanical stimulus associated with solution changes as sequential transfers with HDM did not affect CBF, whereas replacement with HS caused inhibition (Figure 25). Thus, HS appears unable to sustain ciliary activity, suggesting a possible explanation for the lack of cells with actively beating cilia in initial Fura-2 loading experiments.

One major difference in the composition of HS and HDM is the presence of an energy source, in the form of sugar, found only in HDM. To account for this difference, dextrose was added to HS at a concentration corresponding to that present in HDM (5 mM). Long-term (60 min) exposure of ciliated cells to HS supplemented with dextrose (HSD) was examined for maintained ciliary activity. In the majority of cells examined, CBF initially declined, but returned to approximately 80% of pre-wash CBF values by 60 min (Figure 26). This suggests that HSD is able to support normal ciliary activity at 60 min of incubation. In addition, an extremely robust 5-HT response was observed in cells previously incubated in HSD for 60 min (Figure 27). Given that ciliated cells beat at normal rates and retained the ability to respond to 5-HT, HSD was used as the bathing solution in which to perform Fura-2 experiments.

In the presence of HSD, actively beating ciliated cells were reliably found in culture. Therefore, a detailed examination of optimal loading conditions for Fura-2 was initiated. All concentrations of Fura-2-AM (2-20 μM) and incubation times (30-240 min) performed at room temperature resulted in very weakly loaded ciliated cells as determined: (1) qualitatively compared to other non-ciliated cell types; and (2) quantitatively through fluorescence intensity measurements. In comparison, Fura-2 fluorescence intensity in non-ciliated cell types was consistently higher than in any ciliated cell examined (data not shown).

Incubation of ciliated cells in Fura-2-AM (10 μM) at 28°C, in comparison to room temperature incubation (~22°C), resulted in increased fluorescence intensities in the majority of ciliated cells examined. Consistently high fluorescence intensities were recorded in large, granular, actively beating ciliated cells (Figure 28). Thus, isolated cells demonstrating ciliary morphology and activity, and exhibiting a relatively high fluorescence intensity (>60; scale 1-256) throughout the soma were used in all subsequent Fura-2 experiments.

ii) Basal Intracellular Ca^{2+} Concentration

High fluorescence intensity was particularly critical for achieving reliable measurements of intracellular Ca^{2+} concentrations. In initial experiments on weakly-loaded cells, basal intracellular Ca^{2+} concentrations, as determined by using RATIO, appeared to oscillate in a random manner. This result was not corroborated by manual ratio measurements of fluorescence values, or measurements at single excitation wavelengths, using another computer program, NIH Image. In this case, intracellular Ca^{2+} concentration appeared to be stable over time (Figure 29). This discrepancy was abolished when cells having greater levels of Fura-2 fluorescence were used. Thus, reliable measurements of intracellular Ca^{2+} concentrations using RATIO imaging can

only be performed in ciliated cells displaying a background-subtracted fluorescence intensity greater than 60 (excitation at 340 nm with a 0.3 neutral density filter placed in the light path).

Basal intracellular Ca^{2+} concentrations were determined in 4 regions of each ciliated cell; whole cell body, center of the cell, ciliated membrane region, and non-ciliated membrane region (Figure 30B). Ca^{2+} concentrations were similar between regions, maintaining a value around 250 nM (Figure 30A). Moreover, they were stable over the 60 sec time period investigated. Thus, reliable basal Ca^{2+} measurements can be obtained using RATIO on cells meeting the criteria detailed above (see Methods V-iv and Results VI-ii). These results provide the initial platform upon which future calcium imaging experiments utilizing Fura-2 can be consistently performed.

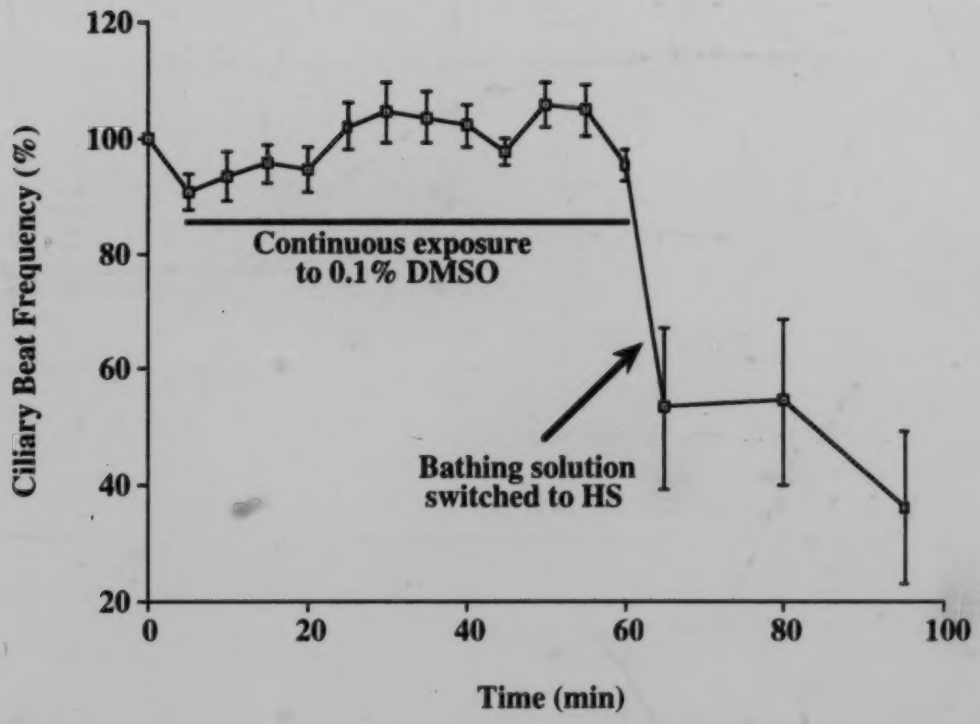
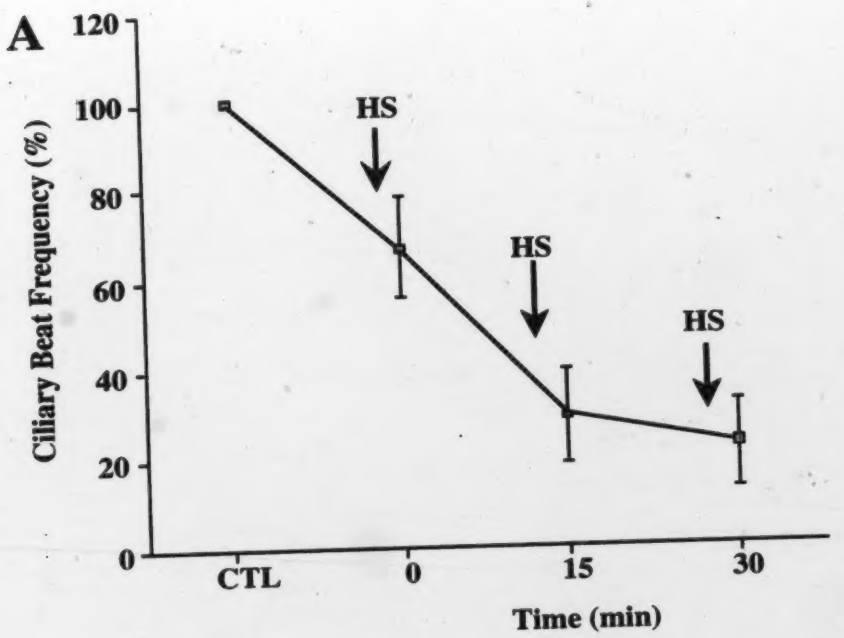
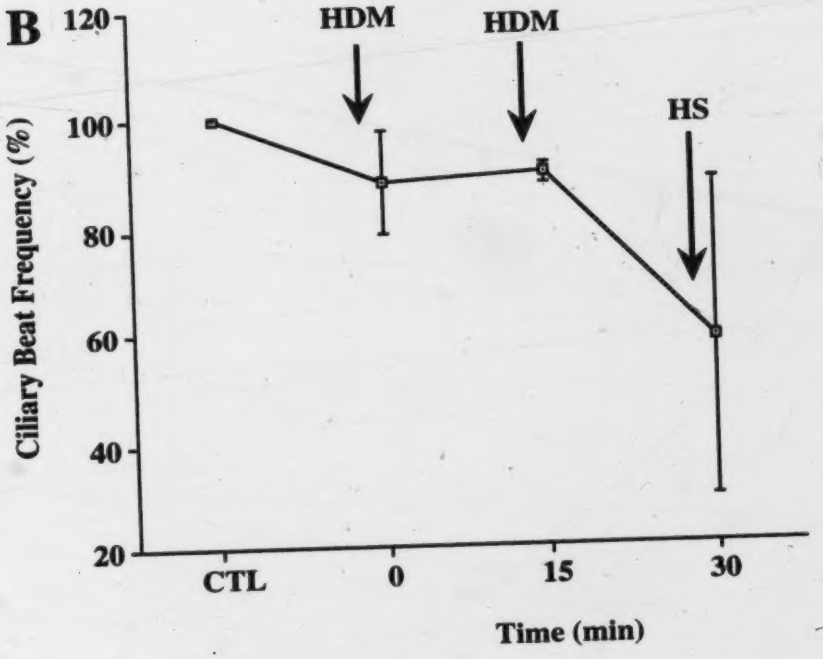


Figure 24: Long-term effects of DMSO and Helisoma saline (HS) on CBF. Incubation of ciliated cells in 0.1% DMSO for 60 min (in HDM) did not affect CBF. However, incubation in HS resulted in a marked, sustained decrease in CBF. Values are normalized to CBF values obtained prior to addition of DMSO. Points are the average response of 8 cells.

Figure 25: Differential effects of *Helisoma* saline and *Helisoma* defined medium on CBF. Values were normalized to CBF recorded in HDM, prior to exchange of bathing solutions (CTL). Solution exchange occurred immediately prior to analysis of CBF. (A) Bathing solution was exchanged with HS every 15 min. Each subsequent exchange resulted in a decrease in CBF. Values are the average response of 13 cells. (B) Bathing solution was exchanged with HDM twice, followed by an exchange with HS. Ciliated cells bathed in HDM did not differ in CBF compared to control (CTL) values. However, CBF decreased after exchange with HS. Values are the average response of 3 cells.


A
120
100
80
60
40
20
0
Ciliary Beat Frequency (%)
HS
HS
HS
CTL
0
15
30
Time (min)


B
120
100
80
60
40
20
Ciliary Beat Frequency (%)
HDM
HDM
HS
CTL
0
15
30
Time (min)

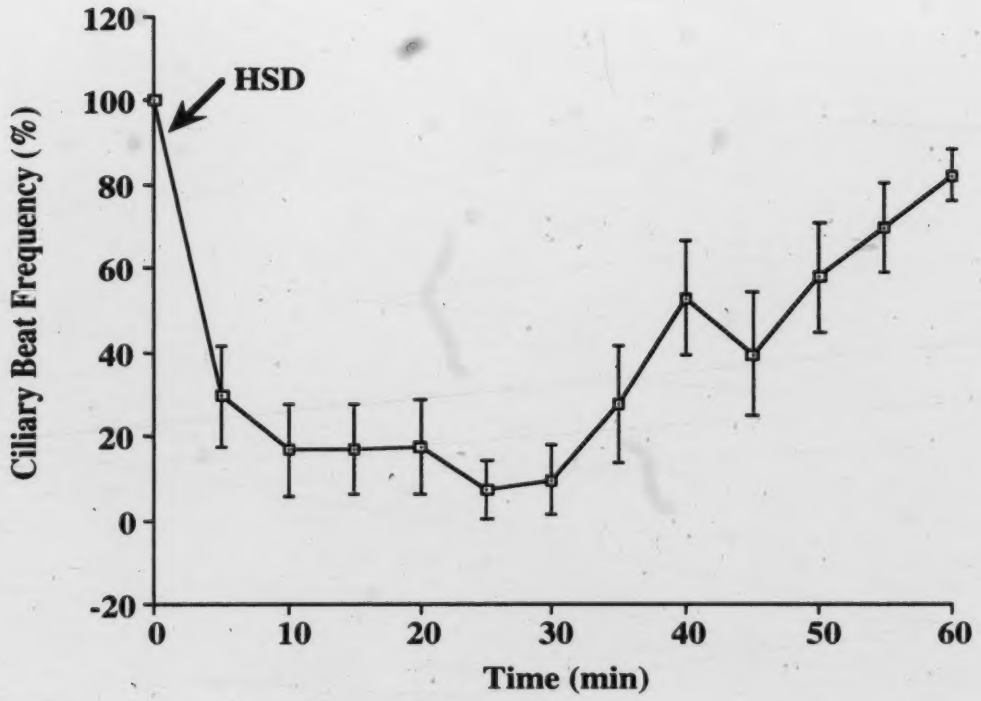


Figure 26: Effect of dextrose-containing HS on CBF. Incubation in HSD (HS supplemented with 5 mM dextrose) caused an initial decrease in CBF, followed by a gradual return to 80% of basal CBF by 60 min. Only ciliated cells beating at the 60 min time point were used in the analysis. Values are normalized to CBF values recorded in HDM, prior to switching the bathing solution to HSD (arrow). Each point represents the mean response of 9 cells.

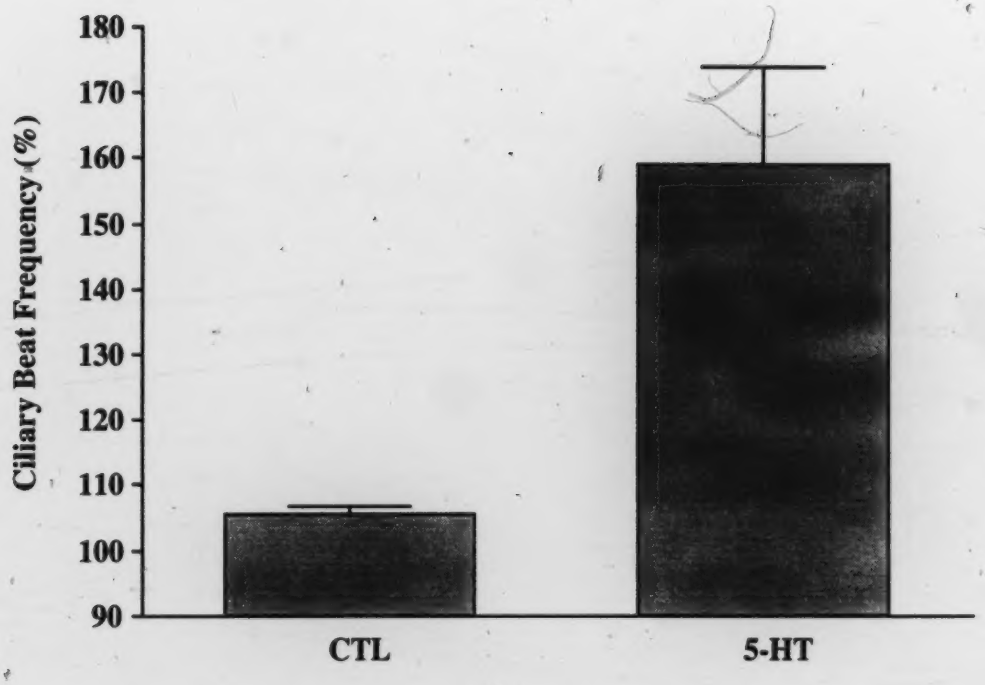


Figure 27: Effect of 5-HT on CBF in ciliated cells bathed in HSD. Ciliated cells were incubated in HSD for 60 min, followed by addition of a HS vehicle control (CTL) or 5-HT (100 μM). 5-HT induced a robust increase in CBF under these conditions. Values are expressed as a percentage increase in CBF compared to CBF values recorded following exposure to HSD for 60 min. Each bar represents the average response of 6 to 9 cells.

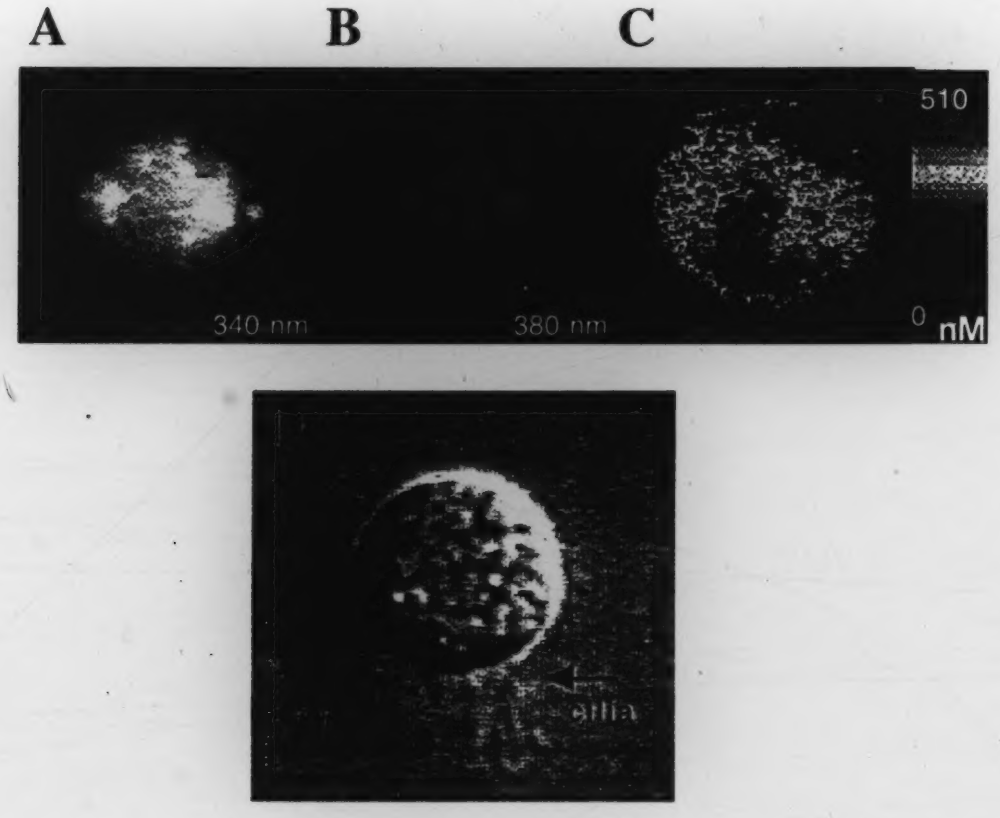


Figure 28: Fura-2 fluorescence in a ciliated cell. Fluorescence excited at 340 nm (A) and 380 nm (B) is shown. False color Ca^{2+} image (C) and Nomarski DIC micrograph (D) of the same cell. Scale bar = 10 µm.

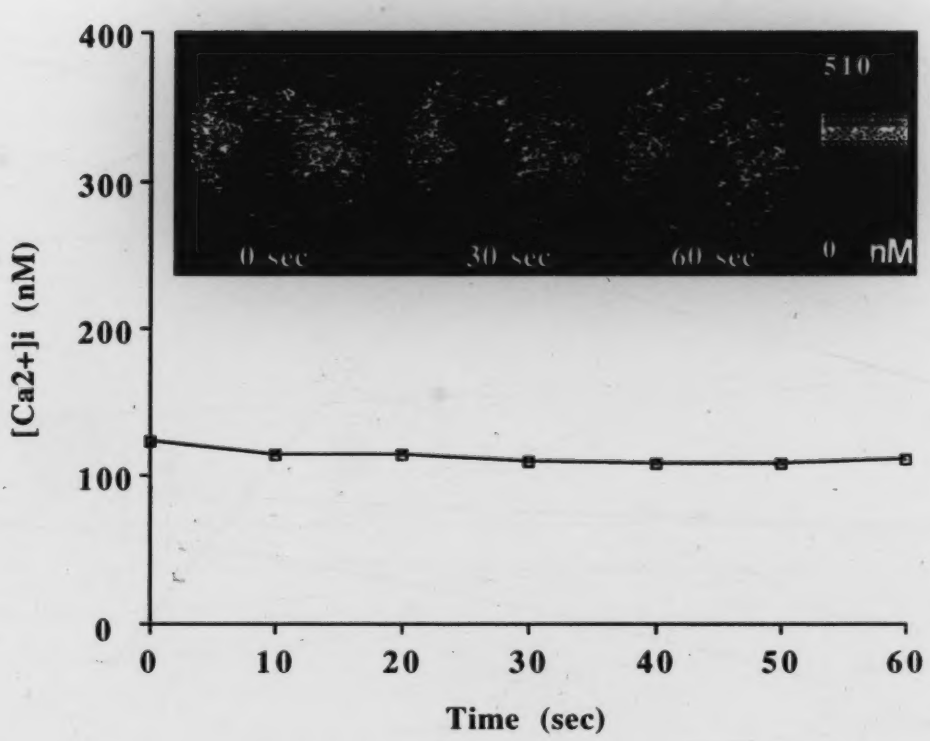
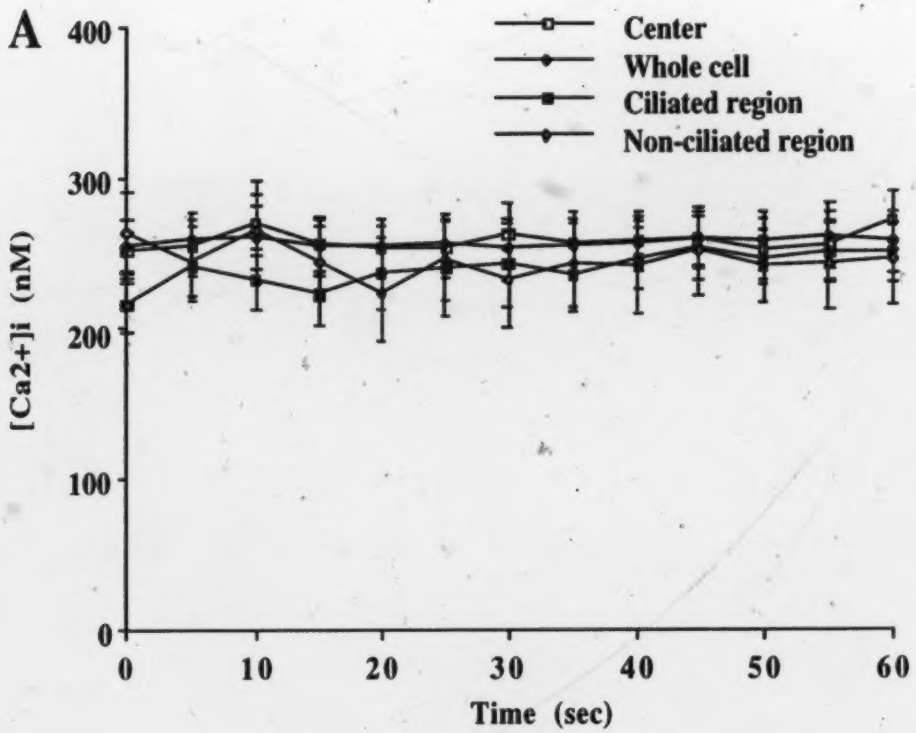
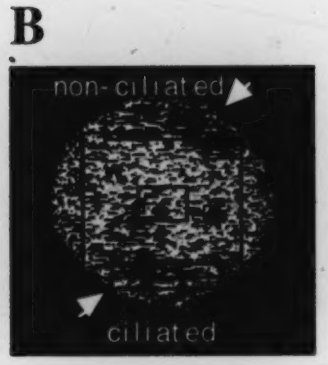


Figure 29: Ca^{2+} concentration remained stable over 60 sec. Concentrations were determined by the computer program NIH Image. Inset: 3 representative false-color images of Ca^{2+} concentrations at specified times after beginning of experiment.

Figure 30: Localized intracellular Ca^{2+} concentrations measured in Fura-2-loaded ciliated cells. Basal intracellular Ca^{2+} measurements (A) were obtained in 4 areas of the cell; center of the cell, (Center), whole cell body (Whole), ciliated membrane region (Ciliated), and non-ciliated membrane region (Non-ciliated). Values are expressed as the average response of 7 cells. Boxes representing each of the 4 areas of the cell (whole cell body, w; center of the cell, c; arrowheads indicate ciliated and non-ciliated regions) are depicted in (B).


A
400
300
200
100
0
[Ca2+]i (nM)
0
10
20
30
40
50
60
Time (sec)
Center
Whole cell
Ciliated region
Non-ciliated region


B
non-ciliated
ciliated

DISCUSSION

I. Ciliary Activity in *Helisoma trivolvis*

Embryos and veligers of gastropod molluscs locomote primarily by cilia-driven swimming behavior (Chia *et al.*, 1984; Diefenbach *et al.*, 1991). Given the small size of these organisms and their poorly developed nervous systems at these early stages, little is known about the physiological mechanisms regulating these behaviors. In recent years, the pond snail *Helisoma trivolvis* has emerged as a valuable model system to address this question. *Helisoma* undergoes direct development from single-cell to juvenile stages within a transparent egg mass over a timecourse of 9-12 days, depending on the temperature of the system. Differentiation of a pair of giant serotonergic neurons, ENC1s, is one of the earliest events in development of the *Helisoma* nervous system. It involves the extension of primary neurites and their extensive branching in the region of the ciliated epithelium of the primordial foot (Goldberg, 1995). During this period of neuronal development (between 20 and 30% of the developmental timecourse; stages E20-E30), embryos also display a tonic, slow cilia-driven rotation behavior that is interrupted by brief periods of accelerated rotation. Pharmacological experiments on whole embryos suggested that these surges of activity result from the enhancement of ciliary beating in response to 5-HT released from ENC1 terminals. Since ENC1 forms both a descending projection to the pedal cilia and an ascending projection that is tipped with a chemosensory-like apparatus at the dorsal embryonic surface (Goldberg and Diefenbach, 1992; Goldberg *et al.*, 1996), it is likely that this serotonergic neuron plays both a sensory and an effector role in regulating ciliary activity.

An important complement to the whole-embryo studies described above is the analysis of embryonic ciliated cells in primary cell culture. The greater experimental accessibility and analytical precision afforded by cell culture facilitates the investigation

of cellular mechanisms. Examination of ciliary beat frequency *in situ* and *in vitro* suggests that normal ciliary activity is maintained in the cell culture environment. Not surprisingly, sustained ciliary beating in culture required the presence of a minimum concentration of sugar (dextrose). However, since increasing the concentration of sugar in the incubating medium did not enhance or hinder basal CBF, regulation of ciliary activity is probably not mediated by alterations in sugar concentrations.

A variety of cell types are found in primary cell cultures of *Helisoma trivolvis* embryos, including nerve cells, fibroblasts, myocytes, and ciliated cells (Goldberg *et al.*, 1988). The ciliated cells are easily distinguished from other cell types based on their rapidly beating cilia. In addition to the ciliated cells examined in this study are flame cells derived from the protonephridia of developing embryos (Christopher *et al.*, 1996). Although flame cells also have rapidly beating cilia, the cilia are exceptionally long (at least 3-4 times longer) and beat with a characteristic flagellar-like waveform. Flame cells were easily identified and were not recorded from in the present study.

Stage E25 embryos, the stage used in all experiments, possess 3 bands of ciliated epithelium, 2 dorsolateral prototrochal bands and a ventromedial pedal band (Diefenbach, 1995). However, when stage E25 embryos are dissociated and placed in cell culture, there are no distinguishing characteristics between pedal and dorsolateral ciliated cells, thus determination of whether any single cell under examination is from the pedal or dorsolateral bands of cilia is virtually impossible. An examination of the beat frequency of the entire population of ciliated cells in culture suggested that the population of cells used in all experiments beat at an average of 8.4 beats per second, slightly higher than the *in situ* beat frequency recorded in isolated embryos (range 4.8 to 7.2 beats per second). This slight difference in beat frequency may be a consequence of the solution in which the measurements were taken. Embryos were isolated from the egg capsule fluid, placed in a *Helisoma* saline/APW solution, immobilized, and beat frequency was recorded. The

lower beat frequency *in situ* may be accounted for by the lack of intracapsular fluid. Alternatively, beat frequency of cultured ciliated cells is measured in defined medium, thus components inherent to this medium may affect ciliary activity. A detailed examination of *in vivo*, *in situ*, and cell culture beat frequencies may lend insight into the cilio-regulatory components inherent to the intracapsular fluid. The slightly higher *in vitro* beat frequencies notwithstanding, the cell culture environment is capable of maintaining ciliary activity. Additionally, cultured ciliated cells maintain a constant basal beat frequency, as no surges or oscillations have been observed (Neumann and Goldberg, unpublished results). Thus, surges, or the transient periods of increased rotation seen in whole embryo behavior studies is not due to inherent surging of the ciliated cells. As 5-HT increased CBF in a concentration-dependent manner both *in vivo* and in cell culture (Diefenbach *et al.*, 1991; Figure 6), the most likely explanation of surge behavior is the pulsatile release of 5-HT from ENC1 neurons onto the ciliated epithelial cells, causing transient increases in ciliary activity and embryo rotation.

Even though the results on basal CBF suggest a homogenous population of ciliated cells, of interest are the cultured ciliated cells that did not respond to 5-HT (100 μM; an increase <120% of basal beat frequency). At the developmental stage used in these experiments, stage E25, only the pedal band of cells is innervated by the serotonergic neurons ENC1 (Diefenbach *et al.*, 1995; Diefenbach, 1995). However, a qualitative analysis of the effect of 5-HT on ciliary activity in whole embryo preparations has revealed that not only do the innervated pedal ciliated cells respond to 5-HT, but the dorsolateral cells also increase their beat frequency when exposed to 5-HT (Diefenbach, unpublished). Thus, it is unlikely that non-responders are dorsolateral ciliated cells. Non-responders may be cells derived from the gut epithelium, which have shorter cilia compared to the locomotory epithelial ciliated cells, as revealed in scanning electron micrographs (McKenney and Goldberg, unpublished results). In the present experiments,

it was commonly found that ciliated cells with relatively short cilia did not respond to 5-HT. An alternative hypothesis is that non-responders are "young" ciliated cells. During development, the number of ciliated cells in the pedal band increases. At stage E25, 6 ciliated cells are present, whereas by stage E30, this number increases to 9 ciliated cells (Diefenbach, 1995). It is possible that non-responders are ciliated cells that have yet to develop the machinery capable of responding to 5-HT. Similar developmentally-dependent regulation of ciliary activity is observed in sea urchin embryos. In sea urchins, the Ca^{2+}-sensitivity of the machinery underlying swimming behavior changes throughout development (Degawa *et al.*, 1986). Thus, non-responders may be ciliated cells derived from the gut epithelium or "young" cells unable to respond to 5-HT as the regulatory machinery has yet to develop.

Previous experiments on cultured ciliated cells suggest that the cilio-excitatory actions of 5-HT are exerted directly on these cells, and not mediated through an intermediary pathway (Goldberg *et al.*, 1994). Furthermore, a pharmacological analysis of the 5-HT receptor mediating this response was completed through complementary whole-embryo and cell culture experiments. The results of this study suggested that the *in vivo* response was accurately recapitulated under cell culture conditions. Since the goal of present study was to study the signal transduction pathway underlying the 5-HT response, which necessarily involves manipulation of the extracellular and intracellular environments, an *in vitro* approach was essential.

II. Comparative Analysis of Ciliary Control

In the commonly studied marine bivalve ***Mytilus edulis***, application of exogenous 5-HT increases ciliary activity in the lateral gill cilia (Murakami, 1983; 1987). This effect recapitulates the endogenous release of 5-HT from the branchial nerve that innervates the gill epithelium (Paparo and Murphy, 1975a). Likewise, in the nudibranch *Tritonia*

diomedea, release of 5-HT from Pedal 21 neurons onto the ciliated foot epithelium increases ciliary activity (Audesirk *et al.*, 1979). Excitation of pedal A-cluster neurons increases ciliary activity of the foot epithelium in a 5-HT-dependent manner in adults of the pulmonate *Lymnaea stagnalis* (Syed and Winlow, 1989). Similar cilio-excitatory effects are observed upon application of exogenous 5-HT to cultured ciliated embryonic ciliated cells from *Helisoma trivolvis* (see Results section II). In this system, 5-HT increases CBF in a concentration-dependent manner and this stimulatory effect is observed within 10 sec of application and is fully reversible. Furthermore, the effect of 5-HT can be significantly reduced by mianserin, a 5-$HT_{2/1C}$ receptor antagonist (Goldberg *et al.*, 1994), suggesting that exogenous 5-HT directly acts upon 5-HT receptors present on the ciliated epithelial cells. Thus, 5-HT-induced cilio-excitation is a common phenomenon observed throughout the molluscan phylum.

Studies on the regulation of ciliary beating in a variety of species have led to the conclusion that multiple signal transduction pathways have evolved to mediate cilio-excitation and cilio-inhibition. Since cyclic AMP and Ca^{2+} appear to be relatively ubiquitous intracellular messengers in ciliary control, sometimes having antagonistic actions (Bergles and Tamm, 1992; Stommel and Stephens, 1985b), they served as the starting point for investigating the cilio-excitatory actions of 5-HT in *Helisoma* embryos. An unexpected finding of the present study was that cyclic AMP does not appear to be involved in regulating ciliary beat frequency. Treatments known to be effective indicators of cyclic AMP-dependent responses (Price and Goldberg, 1993a), such as application of membrane-permeable cyclic-AMP analogs or combined treatment with an adenylate cyclase activator and a phosphodiesterase inhibitor, had no effect on CBF. However, addition of IBMX alone tended to have a weak inhibitory effect on basal CBF. Since the 5-HT-mediated increases in CBF were not affected by increased cyclic AMP concentrations, the IBMX effect suggests that cyclic AMP may play a minor role in the

regulation of basal CBF, but not in 5-HT-mediated cilio-excitation. Perhaps a neurotransmitter other than 5-HT exerts an inhibitory influence on CBF through a cyclic AMP-dependent pathway. Dopamine and octopamine, the only other neurotransmitters tested, do not affect embryo rotation rate (Diefenbach *et al.*, 1991). In contrast to the results on *Helisoma*, cyclic AMP is thought to mediate 5-HT-induced cilio-excitation in the well-studied lateral gill cilia of the marine bivalve *Mytilus edulis* (Murakami, 1983; 1987). Similarly, in *Paramecium*, an elevated intracellular cyclic AMP concentration has cilio-excitatory actions on CBF, which underlies forward swimming behavior (Bonini *et al.*, 1986).

Consistent with many of the previous studies on the regulation of ciliary activity, Ca^{2+} plays a central role in controlling CBF in *Helisoma* embryos. The cilio-excitatory effect of 5-HT was mimicked by treatments that cause an increased intracellular Ca^{2+} concentration and blocked by treatments that prevented the influx of extracellular Ca^{2+}. Thus, Ca^{2+} entry from the extracellular environment, possibly through L-type Ca^{2+} channels, appears to be a crucial step in the signal transduction pathway underlying the 5-HT response.

Ciliary beating appears to have two different modes of regulation in different cell types. In the first, basal frequency can be either up-regulated or down-regulated to increase or decrease CBF. In the second, ciliary beating is primarily under excitatory regulatory control. In most invertebrate and protozoan ciliary systems studied to date, the first mode of beating occurs; that is both cilio-excitation and cilio-inhibition are observed. In these cases, Ca^{2+} is normally involved in mediating inhibitory or reversal responses (Nakaoka *et al.*, 1984; Bergles and Tamm, 1992; Nakumara and Tamm, 1985; Paparo and Murphy, 1975a; 1975b; Murakami and Machemer, 1982). In contrast, cilio-excitatory actions of Ca^{2+} are normally observed in systems that are of the second mode of regulation; that is they are primarily under only excitatory control. For example, the

abfrontal gill cilia of *Mytilus* differ from the lateral gill cilia in that they do not appear to be under positive and negative control by neurotransmitters released from the branchial nerve (Stommel, 1984a). Rather, they respond primarily to mechanostimulation by increasing or initiating ciliary activity in a Ca^{2+}-dependent fashion (Stommel, 1984b). Likewise, cilia found in the respiratory and reproductive tracts of vertebrates are predominantly regulated by mechanical or hormonal excitatory stimuli (Chiyotani *et al.*, 1992; Murakami and Eckert, 1972), with Ca^{2+} playing a role in the cilio-excitatory responses. The cilia examined in the present study conform to this latter type of regulation. The cilia-driven rotation behavior of *Helisoma* embryos does not undergo arrests (Diefenbach *et al.*, 1991), nor is there any known inhibitory control pathway impinging upon the cilia (Diefenbach *et al.*, 1991; Diefenbach, 1995). Thus, the precise role of Ca^{2+} in regulating ciliary activity appears to be determined by functional, rather than evolutionary constraints.

III. Ca^{2+} Regulates Ciliary Activity

i) Pharmacological Manipulation of Intracellular Ca^{2+} Concentrations

Like ciliary beating, neurite outgrowth is regulated to a large extent by intracellular Ca^{2+} concentrations (Goldberg *et al.*, 1992; Kater and Mills, 1991). Increases or decreases in Ca^{2+} concentrations greatly affect the state of growth of the neuron. During neurite outgrowth, Ca^{2+} is maintained at a certain concentration termed the "calcium set-point" (Kater and Mills, 1991; Rehder *et al.*, 1991). Deviations from this set-point will affect Ca^{2+}-dependent pathways within the cell, thus affecting neurite outgrowth and other cellular activities. As Ca^{2+} also appears to be a major regulator of ciliary activity, Ca^{2+} may similarly be maintained around a set point to support ciliary beating. A corollary of this hypothesis is that there exists a specific range of intracellular Ca^{2+} concentrations that supports ciliary beating. For instance, the abfrontal ciliated cells

of *Mytilus edulis* gill were stimulated in response to moderate rises in Ca^{2+} concentration and arrested in response to larger increases (Stommel, 1984b). In the present study, excitation was observed at lower concentrations of KCl, while inhibition was observed at higher depolarizing concentrations. It is not yet known how higher concentrations of KCl induce inhibition of ciliary activity. Perhaps the greater depolarization may induce an increase in intracellular Ca^{2+} beyond a concentration that supports ciliary beating. Furthermore, Ca^{2+}-dependent Ca^{2+} channel inactivation, Ca^{2+} activated potassium channels, or other Ca^{2+} dependent mechanisms may be recruited at specific intracellular Ca^{2+} concentrations and thereby affect ciliary beating.

Ca^{2+} has been demonstrated to be an important regulator of ciliary activity specifically within the ciliary shaft. In embryos of *Mnemiopsis leidyi*, Tamm and Terasaki (1992) demonstrated that voltage-sensitive Ca^{2+} channels are distributed evenly along the entire shaft of the cilium. As KCl would likely activate all membrane-bound channels, the cilio-excitatory and cilio-inhibitory effects of KCl may be directly associated with changes in intraciliary Ca^{2+} concentration, thereby directly affecting the pattern of the ciliary beat.

The molecular mechanism that underlies Ca^{2+} regulation of the ciliary beat is unknown. In many cases, the intracellular effects of Ca^{2+} are mediated by a Ca^{2+} binding protein, such as calmodulin (CaM; *i.e.* neurite outgrowth; Polak *et al.*, 1991). To date, CaM is the only Ca^{2+}-activated molecule that is known to mediate the action of Ca^{2+} on ciliary beating. CaM has been localized to the axoneme in several ciliary preparations (Ohnishi *et al.*, 1982; Stommel *et al.*, 1982; Bergles and Tamm, 1992; Stommel and Stephens, 1985b; Stommel, 1984b). Furthermore, the effect of Ca^{2+} on ciliary activity can be abolished by application of CaM antagonists (Stommel, 1984b; Tamm, 1988; Marsden and Hassessian, 1986; Reed and Satir, 1980; Di Benedetto *et al.*, 1991; Nakaoka *et al.*, 1984). Although the direct role of Ca^{2+}-CaM on the axoneme is unknown,

phosphorylation of axonemal proteins through the action of Ca^{2+}-CaM kinases may be involved in regulating the timing or pattern of dynein-microtubule interactions and thus, the ciliary beat (Satir, 1985; Stommel and Stephens, 1985b; Stommel, 1986).

ii) Calcium Imaging Using Fura-2

A temporal and spatial analysis of intracellular Ca^{2+} concentration using Ca^{2+} imaging techniques was initiated to correlate intracellular Ca^{2+} concentrations and ciliary beating. The Ca^{2+}-sensitive fluorescent indicator, Fura-2, has numerous advantages over its predecessor, quin2 (Grynkiewicz *et al.*, 1985). Fura-2 preferentially binds Ca^{2+} ions with a lower affinity compared to quin2 (Grynkiewicz *et al.*, 1985) and therefore causes less interference with Ca^{2+} actions by lowering available intracellular Ca^{2+}. Since the fluorescence intensity per molecule of Fura-2 is thirty times greater compared to quin2 (Grynkiewicz *et al.*, 1985), less dye must be incorporated into the cell, again resulting in less buffering of transient changes in intracellular Ca^{2+}. Most importantly, Fura-2 is a dual-wavelength ratiometric dye, which provides a greater level of reliability than single wavelength dyes, such as quin2. Two distinct, but vitally important, properties of Fura-2 permit such a ratiometric quantification. First, at each of the excitation wavelengths of light (340 nm and 380 nm), the intensity of emitted fluorescence changes as a function of bound Ca^{2+} (Grynkiewicz *et al.*, 1985; Kao, 1994). Second, in response to various concentrations of Ca^{2+}, the fluorescence emission profile of Fura-2 at 340 nm excitation differs from the fluorescence emission profile at 380 nm excitation (Grynkiewicz *et al.*, 1985; Kao, 1994). Therefore, a ratio measurement of the fluorescence emitted at each wavelength allows the calibration of free intracellular Ca^{2+} concentration based on intensity of fluorescence. Compared to single-wavelength intensity-modulating dyes, ratio measurements reduce artifacts due to differences in dye concentration, excitation light intensity, camera sensitivity, cell thickness, and photobleaching (Grynkiewicz *et al.*,

1985; Kao, 1994; Moore *et al.*, 1990). These factors have similar effects on fluorescence intensities at both wavelengths and therefore cancel in the ratio of the intensities.

The Ca^{2+} imaging equipment was calibrated using Fura-2-containing saline solutions. Several advantages exist for this calibration method, including consistency in the volume of solution used and the concentration of indicator (Fura-2). Furthermore, as several solutions of varying Ca^{2+} concentration can be used, the K_d of Fura-2 for Ca^{2+} can be accurately determined. However, the application of these *in vitro* calibration values to cellular Ca^{2+} measurements is not always valid. Several inherent differences between saline solutions and cell cytoplasm do not permit a direct application of *in vitro* calibration with cellular systems (Moore *et al.*, 1990). For example, the fluorescence properties of the dye may be altered by the viscosity of the intracellular environment or by Fura-2 trapped within intracellular organelles (Moore *et al.*, 1990). Thus, a certain amount of inaccuracy is inherent in the Fura-2 calibration method employed in this study. One popular method used to calibrate Ca^{2+} imaging systems is through use of a population of alike cells loaded with Fura-2. The fluorescence emitted by these cells determined after application of a Ca^{2+} ionophore and after quenching all Ca^{2+} with a Ca^{2+}-chelator, such as EGTA or BAPTA, is used to determine R_{max} and R_{min}, respectively (Kao, 1994). Such calibrating methods could not be used in this study for several reasons. A population of alike cells cannot be obtained using embryo dissociation methods as isolation of ciliated epithelial cells from the numerous other embryonic cell types is virtually impossible. Additionally, dissection of healthy ciliated epithelial cells from stage E25 embryos, independent of surrounding cell types, would be difficult, if not impossible. Due to these reasons, I opted to calibrate the Ca^{2+} imaging system using an *in vitro* method. Although single cell calibration methods do exist, they require cells well loaded with the indicator. As difficulties were encountered obtaining sufficiently high concentrations of Fura-2 within the ciliated cells, a single cell calibration could not be

performed in this study. Additionally, the single cell calibration methods require challenging the cell with very high and very low intracellular Ca^{2+} concentrations; the ability of ciliated cells to withstand such manipulations is unknown. Given the apparent difficulties in obtaining a population of like cells, in addition to the inability to obtain high fluorescence intensities within the ciliated cells, the *in vitro* calibration methods used are appropriate and do provide a valid estimate of the concentration of Ca^{2+} within the cell.

Initial Ca^{2+} imaging experiments were fraught with difficulties in trying to obtain adequate loading of Fura-2. In several other systems studying ciliated cells, similar problems were encountered. Tarasiuk *et al.* (1995) attempted to load frog palate and esophageal cells with the acetoxymethyl ester form of Fura-2 (Fura-2-AM). However, they obtained a high proportion of failures in loading. This problem was overcome with the development of a specific set of criteria for cells exhibiting a high and stable fluorescence when excited at 360 nm. Similarly, in this study, high values of Fura-2 fluorescence intensity were rarely encountered. Additionally, the computer imaging software, RATIO, used to measure Ca^{2+} concentrations does not accurately measure Ca^{2+} at low levels of fluorescence. Thus, a specific criterion for loaded ciliated cells was developed; only cells displaying a relatively high fluorescence intensity (>60) were used in this study. In another study, problems were encountered when attempting to load the ciliate *Didinium nasutum* with Fura-2-AM (Pernberg and Machemer, 1995b). In this case, the acid form of Fura-2 was injected into the ciliate. Such a solution was not implemented in *Helisoma* ciliated cells as these cells are very sensitive to micropipette penetration (Goldberg, Young, and Collins, unpublished results). However, a fast injection system, in which the time required for penetration and injection would be as short as a few seconds, has yet to be attempted.

Ca^{2+} imaging experiments were initiated to examine basal Ca^{2+} concentration within distinct areas of the cell over a specific period of time. Although measurements were made in regions underlying cilia, an examination of intraciliary Ca^{2+} concentrations could not be performed due to the limits in the resolution of the Ca^{2+} imaging equipment. Additionally, a halo of fluorescence emanating from the cell body was observed around the cell, thus hampering the distinction between somal- and ciliary- specific fluorescence (see Figure 28). Measurements of basal Ca^{2+} suggests that all areas of the cell maintain a Ca^{2+} concentration of approximately 200 - 250 nM. Cultured ciliated cells maintain a stable basal CBF, which correlates with a stable basal intrasomal Ca^{2+} concentration. At the whole cell level, these results suggest that the periodicity of the beat cycle is not modulated by changes in intrasomal Ca^{2+}. But, since intraciliary Ca^{2+} could not be measured, specific changes in Ca^{2+} within the cilium may be involved in regulating the distinct phases of the ciliary beat. Also in support of this possibility is that spatial intrasomal Ca^{2+} concentrations remained stable; that is Ca^{2+} concentrations in membrane regions underlying ciliated and non-ciliated regions did not differ from the average Ca^{2+} concentration of the cell body. Thus, basal ciliary beating is not due to varying Ca^{2+} concentrations in distinct areas of the cell. Although "hot-spots" of intracellular Ca^{2+} were occasionally observed (see Figure 28), no systematic examination of these specific regions of higher Ca^{2+} concentration was performed. The physiological relevance of these areas was not examined in this study.

iii) Future Directions in Calcium Imaging

Due to the time taken to establish a Ca^{2+} imaging protocol in ciliated cells, the effect of various stimuli on intracellular Ca^{2+} was not examined in the present study. Results obtained through pharmacological manipulations suggested that increased intracellular Ca^{2+} concentration may be responsible for the observed increases in CBF

(see section III-i). Perifusion of cultured ciliated cells with various concentrations of KCl and determination of the spatial and temporal changes in intracellular Ca^{2+} would help test this hypothesis. In addition, since application of a Ca^{2+} ionophore and thapsigargin increased CBF in a manner similar to 5-HT, it would be useful to examine how each of these treatments affects the intracellular Ca^{2+} concentration.

Several studies have examined the effect of Ca^{2+} within the cilium. Ca^{2+} effectively regulates ciliary activity at the level of the cilium, most likely through interactions with calmodulin (Ohnishi *et al.*, 1982). As the effect of KCl and Ca^{2+} ionophore may occur at the intrasomal or intraciliary levels, changes in Ca^{2+} concentrations within the cilium may underlie the cilio-excitatory effects of 5-HT. However, the equipment presently available does not permit an examination of individual cilia. On the other hand, as thapsigargin also caused an increase in CBF, Ca^{2+} release from intrasomal organelles may underlie 5-HT-stimulated ciliary activity. However, 5-HT-stimulated cilio-excitation was abolished in Ca^{2+}-depleted medium, suggesting that intrasomal Ca^{2+} release is not a primary component of the 5-HT response. It remains that thapsigargin-induced increases in Ca^{2+} may occur spatially near the base of the ciliary shaft, thereby stimulating ciliary activity. The answers to these possibilities may be found through an in-depth examination of spatio-temporal changes in intracellular Ca^{2+} upon manipulation with 5-HT and several other pharmacological agents.

IV. Mechanism of 5-HT-Induced Ca^{2+} Influx

In gastropod molluscs, a number of 5-HT-mediated physiological responses have been examined in considerable detail. A unifying principle that has emerged is that the responses normally involve activation of the cyclic AMP second messenger system. However, the mode of cyclic AMP action and the type of ion channel that is affected is not uniform among different 5-HT responses. Given this trend, it is somewhat surprising

that the cAMP system does not appear to be involved in the 5-HT-induced excitation of ciliary activity. The question thus arises as to how might 5-HT induce the Ca^{2+} influx that appears to be necessary for ciliary activation in these *Helisoma* embryonic cells.

Since the results of the present study directly implicate voltage-gated Ca^{2+} channels in the ciliary response, it follows that Ca^{2+} influx may occur as a consequence of 5-HT-induced depolarization. However, in preliminary electrophysiological experiments on ciliated cells from *Helisoma* embryos, application of 5-HT did not cause a significant change in membrane potential (Collins, Young, and Goldberg, unpublished results). Likewise, the stimulation of *Mytilus* lateral gill cilia by 5-HT does not involve membrane depolarization (Saimi *et al.*, 1983a). In *Helisoma* cells, the increased conductance of voltage-gated Ca^{2+} channels caused by 5-HT may produce a sufficient rise in intracellular Ca^{2+} concentration for ciliary activation, but may be too small to significantly affect membrane potential. In this case, a signal other than membrane depolarization would be acting on the voltage-sensitive Ca^{2+} channels to link 5-HT receptor activation with channel activation. We have previously shown that 5-HT-stimulated cilio-excitation in ciliated cells of *Helisoma trivolvis* embryos is mediated by a novel 5-HT receptor (Goldberg *et al.*, 1994). Although the receptor subtype involved in mediating 5-HT-stimulation of the whole embryo rotation behavior is distinct from all other 5-HT receptors known in vertebrates and invertebrates, it is most closely related to the $5\text{-HT}_{2/1C}$ receptor subtype. This receptor subtype classically works through a G-protein mediated pathway involving formation of IP_3 and DAG, and activation of PKC to increase intracellular Ca^{2+} (Sanders-Bush *et al.*, 1990). In many molluscan systems, 5-HT actions are mediated by PKC-dependent pathways (Hill-Venning and Cottrell, 1992; Sossin *et al.*, 1993; Taussig *et al.*, 1991). For instance, Apl I, one of two forms of PKC that have been identified and cloned in nervous tissue of *Aplysia californica*, underlies 5-HT-stimulated facilitation (Sossin and Schwartz, 1992; Sossin *et al.*, 1993). PKC has also

been shown to enhance Ca^{2+} channel activity in bag cell neurons of *Aplysia californica* (DeRiemer *et al.*, 1985). In addition, a cilio-excitatory role for PKC has recently been demonstrated for ATP-stimulated ciliary activity in frog esophageal tissue (Levin *et al.*, 1997). Likewise, the results presented herein strongly suggest that a PKC-dependent pathway mediates 5-HT-induced cilio-excitation. However, the PKC isoform involved in this response is activated by DAG analogs, but not by phorbol ester application. Thus, I propose that a DAG-dependent, phorbol ester-insensitive isoform of PKC mediates 5-HT stimulated cilio-excitation in embryonic ciliated cells of ***Helisoma trivolvis***.

V. Comparative Analysis of PKC Isoenzymes

Eleven isoforms of PKC have been identified in mammalian tissues (Geiges *et al.*, 1997). These isoforms have been grouped into 3 categories, based on structure and activation profiles; "conventional" Ca^{2+}-dependent PKC subtypes (α, β_1, β_2, and γ), "novel" or "nonconventional" Ca^{2+}-independent PKC subtypes (δ, ε, η, μ, and θ), and "atypical" Ca^{2+}-independent and DAG- or phorbol ester-unresponsive PKC subtypes (ζ and λ) (Geiges *et al.*, 1997).

The evidence presented in this study strongly implicates involvement of PKC in mediating 5-HT-stimulated CBF. However, based on the pharmacological profile, the PKC isoform involved cannot be categorized into any one of the 3 subtypes of PKC. Instead, the data suggest the involvement of a distinct, phorbol ester-insensitive, DAG-dependent isoform possessing properties consistent with all 3 subtypes of PKC. The lack of cilio-excitation upon application of TPA or PDBn suggests categorization of the *Helisoma* PKC isoform into the "atypical" group, as members of this group (ζ and λ) do not bind and are not activated by phorbol esters (Geiges *et al.*, 1997; McGlynn *et al.*, 1992). In contrast, in the present study, DAG analogs specifically stimulated CBF and this effect could be prevented by PKC inhibitors, suggesting DAG-dependent activation

of a PKC pathway. However, the "atypical" PKC isoforms are not activated by DAG (Nakanishi and Exton, 1992), whereas "conventional" and "novel" PKC isoforms are. Taken together, these results strongly suggest that the *Helisoma* PKC isoform displays activation properties consistent with "atypical", "conventional", and "novel" PKC isoform subtypes.

Application of SC-9, a synthetic form of phosphatidylserine, did not mimic 5-HT-induced stimulation. The "atypical" isoform PKCζ displays kinase activity independent of phosphatidylserine alone or phosphatidylserine in the presence of TPA (McGlynn *et al.*, 1992). Likewise, in a related molluscan species, *Aplysia californica*, Apl II, a specific form of PKC found in sensory neurons, was not activated by the application of phosphatidylserine in the presence of Ca^{2+}. However, Apl II possesses sequence homology with the vertebrate PKCε (Kruger *et al.*, 1991; Sossin *et al.*, 1993), a member of the "novel" subtype of PKC. Thus, members of both "atypical" and "novel" PKC isoforms are insensitive to activation by phosphatidylserine. These results, taken together with the results obtained with SC-9, suggest that the *Helisoma* PKC isoform displays activation properties consistent with both the "atypical" and "novel" subtype groups.

Further pharmacological evidence suggests the *Helisoma* PKC isoform may display "conventional" PKC properties. CBF is effectively inhibited by bisindolylmalemide, which is structurally related to staurosporine (Toullec *et al.*, 1991). Staurosporine strongly inhibits Ca^{2+}-dependent, "conventional" PKC isoforms, weakly inhibits Ca^{2+}-independent, "novel" isoforms, and is a very ineffective inhibitor of "atypical" isoforms (Geiges *et al.*, 1997; McGlynn *et al.*, 1992). Therefore, the inhibition of 5-HT-stimulated CBF by bisindolylmalemide strongly suggests that the PKC isoform in this system displays properties inherent to the "conventional" subtype.

A comparative examination of PKC subtypes in a related molluscan species, *Aplysia californica*, with the *Helisoma* PKC isoform provides evidence for interesting

parallels, both functionally and pharmacologically. In nervous tissue of *Aplysia californica*, 2 isoforms of PKC, referred to as Apl I and Apl II, have been identified and cloned (Kruger *et al.*, 1991; Sossin *et al.*, 1993). Apl I shares carboxyl-terminal sequence homology with the vertebrate isoform β_1 and contains a C-2 region, suggesting similarity with the "conventional" subtype of PKC isoforms (Sossin *et al.*, 1993). In *Aplysia*, 5-HT-stimulated facilitation of depressed sensory-to-motor synapses is mediated by activation of Apl I (Sossin and Schwartz, 1992). Likewise, 5-HT stimulation of ciliary activity in *Helisoma* is mediated by PKC, thus, functionally, the neuro-activation of Apl I and *Helisoma* PKC is similar. The second isoform of PKC found in *Aplysia*, Apl II, can be closely aligned with the vertebrate isoform ε, a member of the "novel" subtype. Apl II, unlike Apl I, is not activated by application of phosphatidylserine in the presence of Ca^{2+} (Sossin *et al.*, 1993). Likewise, *Helisoma* PKC is also not activated by phosphatidylserine, suggesting similarities in the pharmacological activation profile of Apl II and *Helisoma* PKC. Thus, a comparative analysis of molluscan PKC isoforms suggests similarities in activation profiles between *Helisoma* and *Aplysia* PKC isoforms. Taken together, the pharmacological activation profile of *Helisoma* PKC shares properties with all 3 subtypes of PKC isoforms.

VI. Transient Activation of CBF by DAG Analogs

The classical method of PKC activation is through DAG, formed by the metabolism of membrane phosphoinositols via the action of phospholipase C (PLC; Sanders-Bush *et al.*, 1990). In addition to phosphatidylinositol metabolism, hydrolysis of phosphatidylcholine by phospholipase D produces phosphatidic acid, which also leads to DAG formation through phosphatidic acid phosphomonoesterase activity (Nishizuka, 1992). In molluscan plasma membranes, the main phospholipids are phosphatidylcholine and phosphatidylethanolamine (Stephens, 1983), thus the action of PLD to produce DAG

and subsequent activation of PKC is likely. Additionally, findings from several systems suggest that phosphatidylinositol hydrolysis is not always required for activation of PKC. Proliferation of the FDCP-Mix 1 stem cell line by interleukin-3 (IL-3) is mediated by PKC activation, but phosphatidylinositol hydrolysis was not observed (Whetton *et al.*, 1988). Similarly, 5-HT-stimulated contraction of guinea pig tracheal muscle is via a PKC pathway. However, phosphoinositide hydrolysis was once again not evident (Watts *et al.*, 1994). Thus, the mechanism underlying DAG formation in 5-HT-stimulated ciliated cells may be a classical PLC pathway or alternatively, a PLD pathway. No attempts to distinguish between the 2 pathways have yet to be undertaken.

Regardless of which phospholipase pathway is involved, 5-HT-induced cilio-excitation was partially mimicked by application of 2 DAG analogs, DiC_8 and OAG. Application of either of these agents resulted in a transient cilio-excitation. Since, application of 5-HT causes a sustained increase in CBF, a DAG-dependent pathway may only partially mediate 5-HT cilio-excitation. Arguments can be made for and against this interpretation. At the concentrations examined and using the extracellular application procedure employed, DAG analogs may produce short-term activation of the PKC cascade, thus resulting in only a transient excitatory effect. Metabolism of the DAG analogs by DAG lipase or DAG kinase may also result in a transient effect. On the other hand, only partial activation of CBF by DAG analogs would occur if 5-HT receptor binding activated yet another signaling pathway to either modulate the activity of DAG/PKC or to sustain CBF through a separate pathway, downstream from PKC activation.

Modulatory effects on PKC pathways have been demonstrated in several systems. In a variety of cell types, including neutrophils, fibroblasts, hepatocytes, and mast cells, phosphatidylinositol-dependent DAG activation of PKC modulates subsequent activation of PKC by DAG formed through phosphatidylcholine metabolism (reviewed in

Nishizuka, 1992). Likewise, *cis*-fatty acids, such as arachidonic, oleic, linoleic, linolenic, and docosahexaenoic, formed by hydrolysis of phosphatidylcholine via non-specific PLA_2, have been demonstrated to be potentiators of DAG-stimulated PKC activation (Nishizuka, 1992; Shinomura *et al.*, 1991). If such potentiating events are activated by 5-HT receptor binding, addition of DAG analogs alone would surely be unable to fully mimic 5-HT-induced cilio-excitation. CBF has been demonstrated to be modulated by arachidonic acid metabolites in other systems (Chiyotani *et al.*, 1992; Weisman *et al.*, 1990), further supporting the possibility that PLA_2 signaling pathways are involved in 5-HT-induced cilio-excitation in the *Helisoma* system.

In contrast to activation of a secondary modulatory effect, 5-HT-receptor binding may activate another signaling pathway separate from PKC. For example, in human (Di Benedetto *et al.*, 1990a; 1991) and rabbit respiratory cilia (Lansley *et al.*, 1992), both cAMP-dependent and Ca^{2+}-dependent pathways regulate ciliary activity. Further, the regulation of *Ciona intestinalis* sperm flagellum suggests a multicomponent kinase cascade is involved in the activation process (Dey and Brokaw, 1991). Sustained ATP-enhanced ciliary activity in frog esophageal tissue is through activation of PKC (Levin *et al.*, 1997). However, the signaling pathway(s), activated prior to PKC, responsible for ciliary activation in frog esophageal tissue is yet unknown. Thus, it is highly probable that signaling pathways, in addition to DAG/PKC, may be responsible for sustained 5-HT ciliary activity in *Helisoma trivolvis* embryos.

Transient effects of DAG analogs may be due to the lack of a concurrent increase in intracellular Ca^{2+}. I have previously demonstrated that 5-HT-stimulated CBF is dependent on an influx of extracellular Ca^{2+} (see Results section IV). The PKC isoform in this system appears to share similarities with conventional isoforms of PKC, whose activation is dependent on Ca^{2+} ions. Thus, without an influx of Ca^{2+}, PKC may only be partially activated by DAG. Experiments using submaximal Ca^{2+} ionophore

concentrations in the presence of DAG analogs would be able to substantiate such a possibility.

VII. PKC Inhibitors Block the 5-HT Response

5-HT-induced cilio-excitation was examined with 2 specific PKC inhibitors, bisindolylmalemide and Calphostin C. When added at concentrations expected to produce specific activity (Toullec *et al.*, 1991; Kobayashi *et al.*, 1989), both compounds significantly reduced cilio-excitation by 5-HT. However, when bisindolylmalemide was tested at a higher concentration (200 nM), it did not abrogate the 5-HT response as it did at lower concentrations (data not shown). However, the specificity of bisindolylmalemide at lower concentrations (100 nM) on PKC inhibition was demonstrated by its effective inhibition of DiC_8-induced cilio-stimulation. At higher concentrations, bisindolylmalemide has been shown to exert non-specific effects on a variety of signal transduction pathways (Toullec *et al.*, 1991). It is possible, therefore, that the paradoxical effect of bisindolylmalemide at 200 nM may be due to its action on additional targets. In any event, as both PKC inhibitors were most effective in blocking the 5-HT response when applied at lower concentrations, where confounding secondary effects are not expected, PKC is strongly implicated in mediating 5-HT-induced cilio-excitation.

VIII. So, What Does 5-HT Do To Ciliated Cells Anyway?

The results presented here suggest that 5-HT-stimulated ciliary activation in early embryos of *Helisoma trivolvis* is due to an influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. In addition, phorbol ester-insensitive, DAG-dependent PKC-mediated pathways also underlie 5-HT-induced cilio-excitation. However, the relationship between Ca^{2+} influx and PKC activation are unknown. PKC has been shown to regulate Ca^{2+} channel activity in some systems (Doerner and Alger, 1992; DeRiemer *et*

al., 1985), thus Ca^{2+} influx may occur subsequent to PKC activation. Ca^{2+}, most likely in concert with CaM, may then act within the soma or cilium to alter CBF. Another possibility is that Ca^{2+} may be required for activation of PKC, as is the case for "conventional" PKC isoforms. PKC activity would then result in phosphorylation of intrasomal or intraciliary proteins to increase CBF. However, the activation profile of *Helisoma* PKC is also similar to "novel" and "atypical" isoforms of PKC, Ca^{2+}-insensitive isoforms, thus Ca^{2+} may not regulate the activity of PKC. A third possibility is that synergistic interactions between elevated Ca^{2+} concentrations and PKC may regulate activity of the ciliary beat cycle. Ca^{2+} may affect the rate of one phase (*i.e.* effective) of the beat cycle, while the action of PKC may affect the rate of the opposite phase (*i.e.* recovery), together causing an increase in CBF. A similar synergistic feedback hypothesis, through activation of kinases and phosphatases, has been proposed for the effects of Ca^{2+} and cAMP underlying the switching mechanism of the beat cycle (Satir, 1985). A final possibility is that Ca^{2+} and PKC may act together at a similar site to affect CBF, possibly through phosphorylation of a single axonemal protein. Further, 5-HT may also activate a number of other signaling pathways, which would act in concert with Ca^{2+} and PKC to regulate ciliary activity. Numerous other ciliary systems have implicated the involvement of signaling pathways, such as arachidonic acid and its metabolites (Chiyotani *et al.*, 1992; Weisman *et al.*, 1990), cyclic GMP (Geary *et al.*, 1995), and nitric oxide (Jain *et al.*, 1993; Sisson, 1995) in regulation of ciliary activity. In fact, a histochemical examination of nitric oxide synthase activity in early embryos of *Helisoma trivolvis* suggests the presence of NOS in the chemosensory structure found at the tip of ENC1 and in the dorsolateral ciliated cells (Cole and Goldberg, unpublished results). As NOS is the enzyme responsible for nitric oxide (NO) production, NO may be formed within the ENC1 soma and released from the terminals onto the pedal ciliated epithelial cells to regulate ciliary activity. Additionally, the observation that dorsolateral cells

contain NOS activity suggests the presence of NO in these cells. Possibly, NO is required in the regulation of ciliary activity in both ciliated cell types, but the dorsolateral cells produce the signaling agent internally, while the pedal cells receive the message through innervation and release from ENC1 terminals. On the other hand, in addition to Ca^{2+} and PKC, NO may be used in dorsolateral cells as a cilio-excitatory signaling agent, whereas a yet unidentified signaling agent may act within the pedal ciliated cells to produce the full response to 5-HT. Evidence supporting the hypothesis that there are diverse signaling pathways within separate populations of ciliated cells from the same tissue comes from the gill of *Mytilus edulis*, where the abfrontal and lateral gill cilia are regulated by different stimuli activating different signaling pathways (Murakami, 1983; 1987; Stommel, 1984a, 1984b).

In embryonic ciliated cells of *Helisoma trivolvis*, 5-HT-stimulated cilio-excitation is mediated by the intracellular signaling pathways, Ca^{2+} and PKC, with no involvement of cAMP. Through use of Ca^{2+} imaging techniques, interactions between and Ca^{2+} and PKC can be thoroughly investigated. Additionally, through pharmacological manipulations and time-lapse videomicroscopy, the involvement of other cilio-regulatory signaling pathways in the 5-HT response can be examined. As the control of ciliary activity is mediated by 5-HT release from ENC1 neurons, this system is ideal to provide the first detailed, integrated understanding of the signaling pathways underlying 5-HT-stimulated ciliary activity.

ENDNOTES

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3. A version of these Results sections has been published. Christopher, K.J., Chang, J.P., and Goldberg, J.I. 1996. Journal of Experimental Biology 199: 1105-1113.
4. A version of this section of the Results has been submitted in abstract form. Christopher, K.J., Young, K.G., Chang, J.P., and Goldberg, J.I. 1997 Society for Neuroscience.

LITERATURE CITED

Aiello, E., Kennedy, J. and Hernandez, C. 1991. Stimulation of frog ciliated cells in culture by acetylcholine and substance P. Comp. Biochem. Physiol. 99C: 497-506.

Ann, K. and Nelson, D. L. 1995. Protein substrates for cGMP-dependent protein phosphorylation in cilia of wild type and *atalanta* mutants of *Paramecium*. Cell Motil. Cytoskel. 30: 252-260.

Arkett, S. A., Mackie, G. O. and Singla, C. L. 1987. Neuronal control of ciliary locomotion in a gastropod veliger (*Calliostoma*). Biol. Bull. 173: 513-526.

Arkett, S. A. 1987. Ciliary arrest controlled by identified central neurons in a urochordate (Ascidiacea). J. Comp. Physiol. A 161: 837-847.

Arkett, S. A., Mackie, G. O. and Singla, C. L. 1989. Neuronal organization of the ascidian (Urochordata) branchial basket revealed by cholinesterase activity. Cell Tissue Res. 257: 285-294.

Audesirk, G., McCaman, R. E. and Willows, A. O. D. 1979. The role of serotonin in the control of pedal ciliary activity by identified neurons in *Tritonia diomedea*. Comp. Biochem. Physiol. 62C: 87-91.

Bargon, J., Trapnell, B. C., Yoshimura, K., Dalemans, W., Pavirani, A., Lecocq, J. and Crystal, R. G. 1992. Expression of the cystic fibrosis transmembrane conductance regulator gene can be regulated by protein kinase C. J. Biol. Chem. 267: 16056-16060.

Bergles, D. and Tamm, S. 1992. Control of cilia in the branchial basket of *Ciona intestinalis* (Ascidiacea). Biol. Bull. 182: 382-390.

Bonini, N. M., Gustin, M. C. and Nelson, D. L. 1986. Regulation of ciliary motility by membrane potential in *Paramecium*: a role for cyclic AMP. Cell Motil. Cytoskel. 6: 256-272.

Bonini, N. M., Evans, T. C., Miglietta, L. A. P. and Nelson, D. L. 1991. The regulation of ciliary motility in *Paramecium* by Ca^{2+} and cyclic nucleotides. In: Advances in Second Messenger and Phosphoprotein Research. New York, Raven Press, Ltd. P. Greengard and G. A. Robison. 227-272.

Bruns, R. F., Miller, F. D., Merriman, R. L., Howbert, J. J., Heath, W. F., Kobayashi, E., Takahashi, I., Tamaoki, T. and Nakano, H. 1991. Inhibition of protein kinase C by Calphostin C is light-dependent. Biochem. Biophys. Res. Comm. 176: 288-293.

Catapane, E. J. 1983. Comparative study of the control of lateral ciliary activity in marine bivalves. Comp. Biochem. Physiol. 75C: 403-405.

Chia, F., Buckland-Nicks, J. and Young, C. M. 1984. Locomotion of marine invertebrate larvae: a review. Can. J. Zool. 62: 1205-1222.

Chiyotani, A., Tamaoki, J., Sakai, N., Isono, K., Kondo, M. and Kondo, K. 1992. Thromboxane A_2 mimetic U46619 stimulates ciliary motility of rabbit tracheal epithelial cells. Prostaglandins 43: 111-120.

Christopher, K. J., Chang, J. P. and Goldberg, J. I. 1996. Stimulation of ciliary beat frequency by serotonin is mediated by a Ca^{2+} influx in ciliated cells of *Helisoma trivolvis* embryos. J. Exp. Biol. 199: 1105-1113.

Cohen, P. 1988. The regulation of phosphorylase kinase activity by calmodulin and troponin. In: Calmodulin. New York, Elsevier Science Publishing Company, Inc. P. Cohen and C. B. Klee. 123-144.

Connor, J. A. 1986. Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian central nervous system cells. Proc. Natl. Acad. Sci. USA 83: 6179-6183.

Corssen, G. and Allen, C. R. 1959. Acetylcholine: its significance in controlling ciliary activity of human respiratory epithelium in vitro. J. Appl. Physiol. 14: 901-904.

Cosson, J. 1992. The covalent oscillator: A paradigm accounting for the sliding/bending mechanism and wave propagation in cilia and flagella. Biol. Cell 76: 319-327.

Degawa, M., Mogami, Y. and Baba, S. A. 1986. Developmental changes in Ca^{2+} sensitivity of sea-urchin embryo cilia. Comp. Biochem. Physiol. 85A: 83-90.

DeLean, A., Munson, P. J. and Rodbard, D. 1978. Simultaneous analysis of families of sigmoidal curves: application of bioassay, radioligand assay, and physiological dose-response curves. Am. J. Physiol. 235: E97-E102.

DeRiemer, S. A., Strong, J. A., Albert, K. A., Greengard, P. and Kaczmarek, L. K. 1985. Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. Nature 313: 313-316.

Dey, C. S. and Brokaw, C. J. 1991. Activation of *Ciona* sperm motility: phosphorylation of dynein polypeptides and effects of a tyrosine kinase inhibitor. J. Cell Sci. 100: 815-824.

Di Benedetto, G., Manara-Shediac, F. S. and Mehta, A. 1990a. The effect of dibutyryl-cyclic AMP on the ciliary activity of human respiratory epithelium *in vitro*. J. Physiol. 422: 36P.

Di Benedetto, G., Manara-Shediac, F. S. and Mehta, A. 1990b. Inhibition of the cyclic AMP-induced rise of ciliary activity by 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine in human respiratory epithelium *in vitro*. J. Physiol. 426: 74P.

Di Benedetto, G., Magnus, C. J., Gray, P. T. A. and Mehta, A. 1991. Calcium regulation of ciliary beat frequency in human respiratory epithelium *in vitro*. J. Physiol. 439: 103-113.

Diefenbach, T. J. and Goldberg, J. I. 1990. Postembryonic expression of the serotonin phenotype in *Helisoma trivolvis*: Comparison between laboratory-reared and wild-type strains. Can. J. Zool. 68: 1382-1389.

Diefenbach, T. J., Koehncke, N. K. and Goldberg, J. I. 1991. Characterization and development of rotational behavior in *Helisoma* embryos: Role of endogenous serotonin. J. Neurobiol. 22: 922-934.

Diefenbach, T. J., Sloley, B. D. and Goldberg, J. I. 1995. Neurite branch development of an identified serotonergic neuron from embryonic *Helisoma*: Evidence for autoregulation by serotonin. Dev. Biol. 167: 282-293.

Diefenbach, T. J. 1995. Development of a serotonergic neuron in embryos of *Helisoma trivolvis*: Developmental and behavioral consequences of serotonin expression. Ph. D. University of Alberta.

Diefenbach, T. J. and Goldberg, J. I. 1995. Developmental and behavioral roles of serotonin from an identified neuron in embryos of the freshwater snail, *Helisoma trivolvis*. Proceedings of the 4th International Congress of Comparative Physiology and Biochemistry. Physiol. Zool. 68: 163.

Doerner, D. and Alger, B. E. 1992. Evidence for hippocampal calcium channel regulation by PKC based on comparison of diacylglycerols and phorbol esters. Brain Res. 597: 30-40.

Eckert, R. and Murakami, A. 1972. Calcium dependence of ciliary activity in the oviduct of the salamander *Necturus*. J. Physiol. 226: 699-711.

Geary, C. A., Davis, C. W., Paradiso, A. M. and Boucher, R. C. 1995. Role of CNP in human airways: cGMP-mediated stimulation of ciliary beat frequency. Am. J. Physiol. 268: L1021-L1028.

Geiges, D., Meyer, T., Marte, B., Vanek, M., Weissgerber, G., Stabel, S., Pfeilschifter, J., Fabbro, D. and Huwiler, A. 1997. Activation of protein kinase C subtypes α, γ, δ, ε, ζ, and η by tumor-promoting and nontumor-promoting agents. Biochem. Pharm. 53: 865-875.

Gibson, I. C. and Logan, S. D. 1992. The actions of phorbol esters upon isolated calcium currents of *Helix aspersa* neurones. Comp. Biochem. Physiol. 102C: 297-303.

Goldberg, J. I., McCobb, D. P., Guthrie, P. B., Lawton, R. A., Lee, R. E. and Kater, S. B. 1988. Characterization of cultured embryonic neurons from the snail *Helisoma*. In: Cell Culture Approaches to Invertebrate Neurosciences. London, Academic Press. D. Beadle, G. Lees and S. B. Kater. 85-108.

Goldberg, J. I. and Kater, S. B. 1989. Expression and function of the neurotransmitter serotonin during development of the *Helisoma* nervous system. Dev. Biol. 131: 483-495.

Goldberg, J. I. and Diefenbach, T. J. 1992. Early development of an identified serotonergic neuron in embryos of the gastropod, *Helisoma trivolvis*. Soc. Neurosci. Abstr. 20: 261.7.

Goldberg, J. I., Mills, L. R. and Kater, S. B. 1992. Effects of serotonin on intracellular calcium in embryonic and adult *Helisoma* neurons. Int. J. Devl. Neurosci. 10: 255-264.

Goldberg, J. I., Koehncke, N. K., Christopher, K. J., Neumann, C. and Diefenbach, T. J. 1994. Pharmacological characterization of a serotonin receptor involved in an early embryonic behavior of *Helisoma trivolvis*. J. Neurobiol. 25: 1545-1557.

Goldberg, J. I. 1995. Neuronal development in embryos of the mollusk, *Helisoma trivolvis*: Multiple roles of serotonin. Advances in Neural Science 2: 67-87.

Goldberg, J. I., Koss, R. and Diefenbach, T. J. 1996. Anatomical evidence of sensory and motor function in early serotonergic neurons from *Helisoma* embryos. Soc. Neurosci. 22: 259.4.

Good, M. J., Stommel, E. W. and Stephens, R. E. 1990. Mechanical sensitivity and cell coupling in the ciliated epithelial cells of *Mytilus edulis* gill. Cell Tissue Res. 259: 51-60.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260: 3440-3450.

Gustin, M. C. and Nelson, D. L. 1987. Regulation of ciliary adenylate cyclase by Ca^{2+} in *Paramecium*. Biochem. J. 246: 337-345.

Hen, R. 1992. Of mice and flies: commonalities among 5-HT receptors. TiPS 13: 160-165.

Hill-Venning, C. and Cottrell, G. A. 1992. Modulation of voltage-dependent calcium current in *Helix aspersa* buccal neurones by serotonin and protein kinase C activators. Exp. Physiol. 77: 891-901.

Iravani, J. and Melville, G. N. 1975. Effects of drugs and environmental factors on ciliary movement. Respiration 32: 157 abstract.

Jain, B., Rubinstein, I., Robbins, R. A., Leise, K. L. and Sisson, J. H. 1993. Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. Biochem. Biophys. Res. Comm. 191: 83-88.

Kao, J. P. Y. 1994. Practical aspects of measuring [Ca^{2+}] with fluorescent indicators. In: Methods in Cell Biology. San Diego, Academic Press. R. Nuccitelli. 155-181.

Kater, S. B. and Mills, L. R. 1991. Regulation of growth cone behavior by calcium. J. Neurosci. 11: 891-899.

Kobayashi, K., Tamaoki, J., Sakai, N., Chiyotani, A. and Takizawa, T. 1988. Inhibition of ciliary activity by phorbol esters in rabbit tracheal epithelial cells. Lung 167: 277-284.

Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. 1989. Calphostin C (UNC-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Comm. 159: 548-553.

Korngreen, A. and Priel, Z. 1994. Simultaneous measurement of ciliary beating and intracellular calcium. Biophys. J. 67: 377-380.

Kruger, K. E., Sossin, W. S., Sacktor, T. C., Bergold, P. J., Beushausen, S. and Schwartz, J. H. 1991. Cloning and characterization of Ca^{2+}-dependent and Ca^{2+}-independent PKCs expressed in *Aplysia* sensory cells. J. Neurosci. 11: 2303-2313.

Lansley, A. B., Sanderson, M. J. and Dirksen, E. R. 1992. Control of the beat cycle of respiratory tract cilia by Ca^{2+} and cAMP. Am. J. Physiol. 263: L232-L242.

Lapetina, E. G., Reep, B., Ganong, B. R. and Bell, R. M. 1985. Exogenous *sn*-1,2-diacylglycerols containing saturated fatty acids function as bioregulators of protein kinase C in human platelets. J. Biol. Chem. 260: 1358-1361.

Levin, R., Braiman, A. and Priel, Z. 1997. Protein kinase C induced calcium influx and sustained enhancement of ciliary beating by extracellular ATP. Cell Calcium 21: 103-113.

Li, X., Giot, J., Kuhl, D., Hen, R. and Kandel, E. R. 1995. Cloning and characterization of two related serotonergic receptors from the brain and the reproductive system of *Aplysia* that activate phospholipase C. J. Neurosci. 15: 7585-7591.

Mackie, G. O., Singla, C. L. and Thiriot-Quievreux, C. 1976. Nervous control of ciliary activity in gastropod larvae. Biol. Bull. 151: 182-199.

Marsden, J. R. and Hassessian, H. 1986. Effects of Ca^{2+} and catecholamines on swimming cilia of the trochophore larva of the polychaete *Spirobranchus giganteus* (Pallas). J. Exp. Mar. Biol. Ecol. 95: 245-255.

Maruyama, I., Yamamoto, T., Ochi, J., Nakai, Y. and Yamada, S. 1983. Dopaminergic innervation and inhibition of ciliary movement in the ciliated epithelium of frog palatine mucosa. Eur. J. Pharmacol. 90: 325-331.

Maruyama, I., Inagaki, M. and Momose, K. 1984. The role of serotonin in mucociliary transport system in the ciliated epithelium of frog palatine mucosa. Eur. J. Pharm. 106: 499-506.

Maruyama, I. 1984. Conflicting effects of noradrenaline on ciliary movement of frog palatine mucosa. Eur. J. Pharmacol. 97: 239-245.

McGlynn, E., Liebetanz, J., Reutener, S., Wood, J., Lydon, N. B., Hofstetter, H., Vanek, M., Meyer, T. and Fabbro, D. 1992. Expression and partial characterization of rat protein kinase C-δ and protein kinase C-ζ in insect cells using recombinant baculovirus. J. Cell. Biochem. 49: 239-250.

McKenney, K. and Goldberg, J. I. 1989. *Helisoma* embryogenesis: Morphological, behavioral and neural development. Soc. Neurosci. Abstr. 15: 1016.

Mogami, Y., Watanabe, K., Ooshima, C., Kawano, A. and Baba, S. A. 1992. Regulation of ciliary movement in sea urchin embryos: Dopamine and 5-HT change the swimming behavior. Comp. Biochem. Physiol. 101C: 251-254.

Moore, E. D. W., Becker, P. L., Fogarty, K. E., Williams, D. A. and Fay, F. S. 1990. Ca^{2+} imaging in single living cells: Theoretical and practical issues. Cell Calcium 11: 157-179.

Mori, M. and Miki-Noumura, T. 1992. Inhibition of gliding movement by calcium in doublet microtubules on *Tetrahymena* ciliary dyneins *in vitro*. Exp. Cell Res. 203: 483-487.

Murakami, A. and Eckert, R. 1972. Cilia: Activation coupled to mechanical stimulation by calcium influx. Science 175: 1375-1377.

Murakami, A. and Machemer, H. 1982. Mechanoreception and signal transmission in the lateral ciliated cells on the gill of *Mytilus*. J. Comp. Physiol. 145: 351-362.

Murakami, A. 1983. Control of ciliary beat frequency in *Mytilus*. J. Submicrosc. Cytol. 15: 313-316.

Murakami, A. 1987. Control of ciliary beat frequency in the gill of *Mytilus* - I. Activation of the lateral cilia by cyclic AMP. Comp. Biochem. Physiol. 86C: 273-279.

Murakami, A. 1989. The control of cilia in metazoa: Ciliary functions and Ca-dependent responses. Comp. Biochem. Physiol. 94A: 375-382.

Nakumara, S. and Tamm, S. L. 1985. Calcium control of ciliary reversal in ionophore-treated and ATP-reactivated comb plates of ctenophores. J. Cell Biol. 100: 1447-1454.

Nakanishi, H. and Exton, J. H. 1992. Purification and characterization of the ζ isoform of protein kinase C from bovine kidney. J. Biol. Chem. 267:

Nakaoka, Y., Tanaka, H. and Oosawa, F. 1984. Ca^{2+}-dependent regulation of beat frequency of cilia in *Paramecium*. J. Cell Sci. 65: 223-231.

Nishino, H., Kitagawa, K., Iwashima, A., Ito, M., Tanaka, T. and Hidaka, H. 1986. N-(6-Phenylhexyl)-5-chloro-1-naphthalenesulfonamide is one of a new class of activators for Ca^{2+}-activated, phospholipid-dependent protein kinase. Biochimica et Biophysica Acta 889: 236-239.

Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258: 607-614.

Ohnishi, K., Suzuki, Y. and Watanabe, Y. 1982. Studies on calmodulin isolated from *Tetrahymena* cilia and its localization within the cilium. Exp. Cell Res. 137: 217-227.

Paparo, A. A. and Murphy, J. A. 1975a. The effect of Ca on the rate of beating of lateral cilia in *Mytilus edulis* - I. A response to perfusion with 5-HT, DA, BOL and PBZ. Comp. Biochem. Physiol. 50C: 9-14.

Paparo, A. A. and Murphy, J. A. 1975b. The effect of Ca on the rate of beating of lateral cilia in *Mytilus edulis* - II. A response to electrical stimulation of the branchial nerve. Comp. Biochem. Physiol. 50C: 15-19.

Paparo, A. A. 1986. Neuroregulatory activities and potassium enhancement of lateral ctenidial beating in *Crassostrea virginica*. Comp. Biochem. Physiol 84A: 585-588.

Pernberg, J. and Machemer, H. 1995a. Voltage-dependence of ciliary activity in the ciliate *Didinium nasutum*. J. Exp. Biol. 198: 2537-2545.

Pernberg, J. and Machemer, H. 1995b. Fluorometric measurement of the intracellular free Ca^{2+}-concentration in the ciliate *Didinium nasutum* using Fura-2. Cell Calcium 18: 484-494.

Pizzi, M., Galli, P., Consolandi, O., Arrighi, V., Memo, M. and Spano, P. F. 1996. Metabotropic and ionotropic transducers of glutamate signal inversely control cytoplasmic Ca^{2+} concentration and excitotoxicity in cultured cerebellar granule cells: Pivotal role of protein kinase C. Mol. Pharm. 49: 586-594.

Polak, K. A., Edelman, A. M., Wasley, J. W. F. and Cohan, C. S. 1991. A novel calmodulin antagonist, CGS 9343B, modulates calcium-dependent changes in neurite outgrowth and growth cone movements. J. Neurosci. 11: 534-542.

Price, C. J. and Goldberg, J. I. 1993a. Serotonin activation of a cyclic AMP-dependent sodium current in an identified neuron from *Helisoma trivolvis*. J. Neurosci. 13: 4979-4987.

Price, C. J. and Goldberg, J. I. 1993b. Pharmacological properties of a serotonin-activated sodium current in an identified neuron from *Helisoma trivolvis*. Soc. Neurosci. 19: 527.1.

Reed, W. and Satir, P. 1980. Calmodulin in mussel gill epithelial cells: Role in ciliary arrest. Annu. N.Y. Acad. Sci. 356: 423-426.

Rehder, V., Jensen, J. R., Dou, P. and Kater, S. B. 1991. A comparison of calcium homeostasis in isolated and attached growth cones of the snail *Helisoma*. J. Neurobiol. 22: 499-511.

Rehder, V. and Kater, S. B. 1992. Regulation of neuronal growth cone filopodia by intracellular calcium. J. Neurosci. 12: 3175-3186.

Saimi, Y., Murakami, A. and Takahashi, K. 1983a. Electrophysiological correlates of nervous control of ciliary arrest response in the gill epithelial cells of *Mytilus*. Comp. Biochem. Physiol. 74A: 499-506.

Saimi, Y., Murakami, A. and Takahashi, K. 1983b. Ciliary and electrical responses to intracellular current injection in the ciliated epithelium of the gill of *Mytilus*. Comp. Biochem. Physiol. 74A: 507-511.

Salathe, M., Pratt, M. M. and Wanner, A. 1993. Protein kinase C-dependent phosphorylation of a ciliary membrane protein and inhibition of ciliary beating. J. Cell Sci. 106: 1211-1220.

Salathe, M. and Bookman, R. J. 1995. Coupling of $[Ca^{2+}]i$ and ciliary beating in cultured tracheal epithelial cells. J. Cell Sci. 108: 431-440.

Sale, W. S. and Satir, P. 1977. Direction of active sliding of microtubules in *Tetrahymena* cilia. Proc. Natl. Acad. Sci. USA 74: 2045-2049.

Sanders-Bush, E., Tsutsumi, M. and Burris, K. D. 1990. Serotonin receptors and phosphatidylinositol turnover. Annu. N.Y. Acad. Sci. 600: 224-236.

Sanderson, M. J., Lansley, A. B. and Dirksen, E. R. 1992. Regulation of ciliary beat frequency in respiratory tract cells. Chest 101: 69S-71S.

Satir, P. 1975. Ionophore-mediated calcium entry induces mussel gill ciliary arrest. Science 190: 586-588.

Satir, P., Wais-Steider, J., Lebduska, S., Nasr, A. and Avolio, J. 1981. The mechanochemical cycle of the dynein arm. Cell Motility 1: 303-327.

Satir, P. 1982. Mechanisms and controls of microtubule sliding in cilia. In: Prokaryotic and Eukaryotic Flagella; Symposia of the Society for Experimental Biology. Cambridge, Cambridge University Press. 179-201.

Satir, P. 1985. Switching mechanisms in the control of ciliary motility. In: Modern Cell Biology. New York, Alan R. Liss, Inc. B. H. Satir. 1-46.

Satir, P. and Matsuoka, T. 1989. Splitting the ciliary axoneme: Implications for a "switch-point" model of dynein arm activity in ciliary motion. Cell Motil. Cytoskel. 14: 345-358.

Satir, P. 1992. Mechanisms of ciliary movement: contributions from electron microscopy. Scanning Microscopy 6: 573-579.

Schultz, J. E., Schönefeld, U. and Klumpp, S. 1983. Calcium/calmodulin-regulated guanylate cyclase and calcium-permeability in the ciliary membrane from *Tetrahymena*. Eur. J. Biochem. 137: 89-94.

Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. and Nishizuka, Y. 1991. Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: Its possible implications. Proc. Natl. Acad. Sci. USA 88: 5149-5153.

Sisson, J. H. 1995. Ethanol stimulates apparent nitric oxide-dependent ciliary beat frequency in bovine airway epithelial cells. Am. J. Physiol. 268: L596-L600.

Sleigh, M. A. and Barlow, D. I. 1982. How are different ciliary beat patterns produced? In: Prokaryotic and Eukaryotic Flagella; Symposia of the Society for Experimental Biology. Cambridge, Cambridge University Press. 140-157.

Soliman, S. 1983. Pharmacological control of ciliary activity in the young sea urchin larva. Effects of monaminergic agents. Comp. Biochem. Physiol. 76C: 181-191.

Sossin, W. S., Diaz-Arrastia, R. and Schwartz, J. H. 1993. Characterization of two isoforms of protein kinase C in the nervous system of *Aplysia californica*. J. Biol. Chem. 268: 5763-5768.

Sossin, W. S. and Schwartz, J. H. 1992. Selective activation of Ca^{2+}-activated PKCs in *Aplysia* neurons by 5-HT. J. Neurosci. 12: 1160-1168.

Stephens, R. E. 1983. Reconstitution of ciliary membranes containing tubulin. J. Cell Biol. 96: 68-75.

Stommel, E. W., Stephens, R. E., Masure, H. R. and Head, J. F. 1982. Specific localization of scallop gill epithelial calmodulin in cilia. J. Cell Biol. 92: 622-628.

Stommel, E. W. 1984a. Calcium regenerative potentials in *Mytilus edulis* gill abfrontal ciliated epithelial cells. J. Comp. Physiol. A 155: 445-456.

Stommel, E. W. 1984b. Calcium activation of mussel gill abfrontal cilia. J. Comp. Physiol. A 155: 457-469.

Stommel, E. W. and Stephens, R. E. 1985a. Calcium-dependent phosphatidylinositol phosphorylation in lamellibranch gill lateral cilia. J. Comp. Physiol. A 157: 441-449.

Stommel, E. W. and Stephens, R. E. 1985b. Cyclic AMP and calcium in the differential control of *Mytilus* gill cilia. J. Comp. Physiol. A 157: 451-459.

Stommel, E. W. 1986. Mechanical stimulation activates beating in calcium-arrested lateral cilia of *Mytilus edulis* gill. J. Muscle Res. Cell Motil. 7: 237-244.

Sugamori, K. S., Sunahara, R. K., Guan, H.-C., Bulloch, A. G. M., Tensen, C. P., Seeman, P., Niznik, H. B. and Van Tol, H. H. M. 1993. Serotonin receptor cDNA cloned from *Lymnaea stagnalis*. Proc. Natl. Acad. Sci. USA 90: 11-15.

Syed, N. I. and Winlow, W. 1989. Morphology and electrophysiology of neurons innervating the ciliated locomotor epithelium in *Lymnaea stagnalis* (L.). Comp. Biochem. Physiol. 93A: 633-644.

Tamaoki, J., Kondo, M. and Takizawa, T. 1989. Effect of cAMP on ciliary function in rabbit tracheal epithelial cells. J. Appl. Physiol. 66: 1035-1039.

Tamm, S. L. 1988. Calcium activation of macrocilia in the ctenophore *Beroë*. J. Comp. Physiol. A 163: 23-31.

Tamm, S. L. and Terasaki, M. 1994. Visualization of calcium transients controlling orientation of ciliary beat. J. Cell Biol. 125: 1127-1135.

Tarasiuk, A., Bar-Shimon, M., Gheber, L., Korngreen, A., Grossman, Y. and Priel, Z. 1995. Extracellular ATP induces hyperpolarization and motility stimulation of ciliary cells. Biophys. J. 68: 1163-1169.

Tash, J. S. 1989. Protein phosphorylation: The second messenger signal transducer of flagellar motility. Cell. Motil. Cytoskel. 14: 332-339.

Taussig, R., Sweet-Cordero, A. and Scheller, R. H. 1989. Modulation of ionic currents in *Aplysia* motor neuron B15 by serotonin, neuropeptides, and second messengers. J. Neurosci. 9: 3218-3229.

Thastrup, O., Cullen, P. J., Drøbak, B. K., Hanley, M. R. and Dawson, A. P. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA 87: 2466-2470.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovsky, J. 1991. The bisindolylmalemide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. 266: 15771-15781.

Verdugo, P., Johnson, N. T. and Tam, P. Y. 1980. β-adrenergic stimulation of respiratory ciliary activity. J. Appl. Physiol. 48: 868-871.

Walczak, C. E. and Nelson, D. L. 1994. Regulation of dynein-driven motility in cilia and flagella. Cell Motil. Cytoskel. 27: 101-107.

Watts, S. W., Cox, D. A., Johnson, B. G., Schoepp, D. D. and Cohen, M. L. 1994. Contractile serotonin-2A receptor signal transduction in guinea pig trachea: Importance of protein kinase C and extracellular and intracellular calcium but not phosphoinositide hydrolysis. J. Pharm. Exp. Ther. 271: 832-844.

Weisman, Z., Fink, A., Alon, A., Poliak, Z., Tabachnik, E., Priscu, L. and Bentwich, Z. 1990. Leukotriene C4 decreases the activity of respiratory cilia *in vitro*. Clin. Exp. Allergy 20: 389-393.

Weiss, T., Gheber, L., Shoshan-Barmatz, V. and Priel, Z. 1992. Possible mechanism of ciliary stimulation by extracellular ATP: involvement of calcium-dependent potassium channels and exogenous Ca^{2+}. J. Membrane Biol. 127: 185-193.

Whetton, A. D., Monk, P. N., Consalvey, S. D., Huang, S. J., Dexter, T. M. and Downes, C. P. 1988. Interleukin 3 stimulates proliferation via protein kinase C activation without increasing inositol lipid turnover. Proc. Natl. Acad. Sci. USA 85: 3284-3288.

Williams, D. A. and Fay, F. S. 1990. Intracellular calibration of the fluorescent calcium indicator Fura-2. Cell Calcium 11: 75-83.

Witman, G. B. 1990. Introduction to cilia and flagella. In: Ciliary and Flagellar Membranes. New York and London, Plenum Press. R. A. Bloodgood. 1-30.